THE RELATIONSHIP OF LIPID AND PROTEIN SYNTHESIS TO MITOCHONDRIOGENESIS IN YEAST

by

Paul A. GORDON

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Statement

The results described in this thesis were obtained by myself under the supervision of Dr Peter R. Stewart. Results obtained in collaboration with others are specifically acknowledged.

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Synopsis

This thesis records the results of investigations of aspects of lipid and protein syntheses in the facultatively anaerobic yeast *Saccharomyces cerevisiae*. The studies presented have been directed towards an understanding of the biogenesis of yeast mitochondria. For this reason emphasis has been placed on the control of synthesis and assembly of mitochondrial lipids and proteins, although attempts have been made to relate these events to the economy of the whole cell. The lipids examined have been phospholipid, fatty acids (particularly unsaturated fatty acids), ergosterol, and ubiquinone. Protein synthesis in mitochondrial and cytoplasmic compartments of the cell has been measured using both *in vivo* and *in vitro* techniques. Cytochrome and RNA content, respiration, and the activities of various mitochondrial enzymes have also been measured. The main results of this investigation are outlined below.

1. Ubiquinone has been assayed as a specifically mitochondrial, non-protein component of the respiratory chain. Cellular levels of ubiquinone are extremely sensitive to anaerobiosis and catabolite repression : the environmental control of ubiquinone in these respects is similar to that of enzymic components of the respiratory chain. Nevertheless it is possible to show that under some conditions the synthesis and assembly of ubiquinone can be completely dissociated from the synthesis and assembly of the mitochondrial enzymes.

2. Comparison has been made of the biochemical properties of *S. cerevisiae* grown anaerobically with and without essential lipids (unsaturated fatty acids and ergosterol), and the behaviour of these cell types during subsequent aeration. It was found that:

(a) lipid-supplemented anaerobes retain the activity of the mitochondrial and cytoplasmic protein-synthesising systems, while these are lost in depleted anaerobes. This is true for both *in vivo* and *in vitro* protein synthesis;

(b) whole cell RNA synthesis is also affected by lipid depletion : the consequence of this seems to be a specific loss of mitochondrial ribosomal RNA.

3. Aeration of lipid-depleted anaerobes results in the re-establishment of the mitochondrial and cytoplasmic protein-synthesising systems. As well, there are rapid, lag-free syntheses of mitochondrial respiratory enzymes and lipids, accompanied by the development of respiratory activity. The aeration system was used to detect and examine interregulatory mechanisms existing between the various induced syntheses.

4. Under some experimental situations it appears that lipid synthesis is partly coupled to protein synthesis. This coupling could be demonstrated by the inhibition of the induced lipid syntheses (that occurred during aeration of lipid-depleted anaerobes) by protein synthesis inhibitors. The inhibition of induced lipid syntheses was not due to non-specific effects of the antibiotics used. Also, the coupling is not present in petite cells.

5. From studies on an unsaturated fatty acid auxotroph, KD115, it is concluded that mitochondrial protein synthesis and cytoplasmic protein synthesis, under aerobic conditions, is dependent on the availability of unsaturated fatty acids. However under some conditions this dependency is not evident.

6. The results indicate that there is a complex set of inter-relationships between the synthesis of mitochondrial proteins, on one hand, and mitochondrial lipids on the other. The types of control mechanisms that affect the synthesis of mitochondrial enzymes are discussed. With these in mind, an interpretation is presented where the protein synthesis-lipid synthesis coupling is dependent on the activity of the mitochondrial protein-synthesising system.

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CHAPTER I

GENERAL INTRODUCTION

1. PROBLEMS ASSOCIATED WITH ORGANELLE DEVELOPMENT

Cell membranes, because of their fundamental importance in biology, have been the focus of many biochemical studies. A large section of these studies has been concerned with the membrane systems of organelles such as chloroplasts and mitochondria. Some of the questions raised by these studies are considered below, with the field of organelle development in mind.

Problems relating to organelle development have been considered from two general viewpoints. Firstly, many studies have been aimed at defining the molecular composition of membranes, the spatial organisation of the membrane lattice, and the relationship of these to membrane function. As a result of this work many membrane models have been proposed and these are discussed later in this chapter. The second viewpoint has emphasised the integrated synthesis and assembly of elements of functional membranes during cell growth and division. Central to this type of study is an examination of the sites of synthesis of the individual membrane components, and the control of the rates of these syntheses. The knowledge thus gained is basic to an understanding of the assembly of components such as lipids and proteins into various types of membrane. With the discovery of organelle DNA, and the associated question of the biological significance of this DNA, there has been much debate on the problem of organelle autonomy. For example, to what extent can the organelle code for and synthesise its own components? Again, this problem is related to that concerning the cellular site of synthesis of organelle components.

At this time there are very few definite answers to the questions raised, although a great deal of effort continues to be expended. The review presented below describes aspects that have been considered in this thesis, deals with recent advances made in other laboratories, and is designed to give some perspective to the experiments to be described later. The present study considers questions relating to the synthesis of lipids and proteins and their assembly into the mitochondrial membranes of the yeast *Saccharomyces cerevisiae*. The results have been considered firstly as part of the problem of how functional mitochondria are made in the cell, and secondly as part of the more general problem of membrane biogenesis. As this study describes work carried out on mitochondria, in particular yeast mitochondria, it is worthwhile considering some of the advantages that such an experimental system offers.

2. THE USE OF YEAST IN THE STUDY OF MITOCHONDRIAL BIOGENESIS.

Ideally, the type of study suggested above requires an experimental system which can be subjected to considerable manipulation. The physiology of more advanced eucaryotic organisms can only tolerate limited change in respiratory function. These organisms are 'obligate' aerobes and must maintain functional mitochondria. Also these organisms, which include mammals, are often under homeostatic controls which tend to buffer any change in the environment. These factors make more advanced organisms difficult systems in which to study membrane biogenesis.

However, a highly useful system is provided by the facultative anaerobe, *S. cerevisiae*. Because this organism, one of the least advanced eucaryotes, can function without mitochondrial respiratory function, the degree to which the development of this organelle can be manipulated is virtually unlimited. In addition, the use of a unicellular organism such as yeast gives the advantages of a short division time, growth on a chemically-defined medium, and relatively simple

and defined methods of genetic analysis. Yeast mitochondria also appear to be structurally and functionally very similar to those of mammals (see, for example, Lloyd, 1969) so it is likely that they provide a good model system. Before considering the factors that control the formation of yeast mitochondria, some of the biosynthetic mechanisms relating to mitochondrial biogenesis will be discussed.

3. MITOCHONDRIAL BIOGENESIS.

(a) The origin of mitochondria and mitochondrial autonomy.

Early ideas on the origin of mitochondria were related to the problem of how eucaryotic organisms arose from more primitive procaryotes. It was suggested that chloroplasts and mitochondria were originally algal and bacterial endosymbionts, respectively, in protozoan cells (for reviews of early literature see Novikoff, 1961; Lehninger, 1964). More recently these ideas have been developed to include the biochemical similarities of organelles and procaryotes (for example, Sagan, 1967; Roodyn and Wilkie, 1968; Lloyd, 1969; Nass, 1969a; Ashwell and Work, 1970; Raven, 1970; and an interesting discussion by Broda, 1970). As these ideas have been amply documented, it is only proposed to give a brief summary here.

Suggestions that mitochondria originated from procaryotic organisms imply, of course, that these organelles may have a considerable degree of autonomy. Evidence relating to mitochondrial autonomy bears closely on the problem of the origin of mitochondria.

(i) The extent of mitochondrial autonomy.

Present evidence clearly shows that mitochondria from all species examined have their own protein-synthesising system, different from the cytoplasmic system, and characterized by distinct transfer and ribosomal RNA species. Mitochondria

also contain a characteristic DNA species, with associated DNA-dependent DNA polymerase and DNA-dependent RNA polymerase. The presence of these systems is suggestive of a considerable degree of autonomy. Genetic evidence also indicates that mitochondrial formation is under the control of extranuclear genes which show maternal rather than Mendelian inheritance, and the suggestion is that these are located on mitochondrial DNA.

It is remarkable that mitochondria and bacteria are similar in many respects. They are about the same size, their protein-synthesising systems are sensitive to similar antibiotics, both contain membrane-bound systems of coupled oxidation and phosphorylation, and DNA from both is generally circular. These findings have been taken to support the 'symbiosis' hypothesis, and as indirect evidence of mitochondrial autonomy. In an interesting experimental approach to the question of organelle autonomy, Nass (1969b) has reported the uptake of isolated chloroplasts into the cytoplasm of phagocytic mouse fibroblasts in tissue culture : the chloroplasts maintained their integrity and were found to remain active over a period of at least two days. Mitochondria from chicken liver were also found to be incorporated into the fibroblast cells. Possibly these experiments provide a model system that mimics an ancient evolutionary pattern.

(ii) Evidence against organelle autonomy. The limited biosynthetic capacity of mitochondria.

Calculation shows that mitochondrial DNA has a very limited coding capacity. There are enough cistrons perhaps for about 30-100 proteins of 20,000 molecular weight, and these are certainly not sufficient for the entire complement of proteins of the complete organelle. Genetic evidence supports a limited role for the mitochondrial genome in coding for mitochondrial proteins, since several classes of nuclear mutations are known which affect mitochondrial proteins.

Furthermore, recent studies have shown that many mitochondrial proteins are synthesised on cytoplasmic ribosomes. This has been particularly well-established for cytochrome c and ATPase (F_1), both of which are synthesised independent of the function of the mitochondrial protein-synthesising system (for example, Kadenback, 1967; Gonzalez - Cadavid and Campbell, 1967; Tzagoloff, 1969a, b). These studies raise questions concerning the nature of the product(s) of mitochondrial protein synthesis. It is proposed on the basis of both *in vitro* and *in vivo* experiments that only inner membrane 'insoluble' protein is synthesised by the mitochondrial protein-synthesising system (Haldar, Freeman and Work, 1966; Beattie, 1968; Beattie, Basford and Koritz, 1966, 1967; Neupert, Brdiczka and Bücher, 1967) and that other classes of mitochondrial protein (outer membrane protein, soluble protein, remaining protein of the inner membrane) are made on the cytoplasmic ribosomes and transferred into the mitochondria (Kadenback, 1967; Gonzalez-Cadavid and Campbell, 1967; Beattie, 1968) at a subsequent step. Yang and Criddle (1970) have recently fractionated the membrane proteins of yeast mitochondria after *in vitro* labelling. Their study revealed a membrane protein fraction the labelling of which was sensitive to chloramphenicol inhibition and glucose repression, indicating that this fraction was the product of the mitochondrial protein-synthesising system. However, the definition of 'inner-membrane insoluble' proteins or membrane protein remains vague, although it has been suggested that these might be the 'structural' protein described by earlier workers (Criddle, Bock, Green and Tisdale, 1962; Richardson, Hultin and Fleischer, 1964). These suggestions have done little but cloud the issue, particularly as the status of 'structural' protein has been seriously questioned (Schnaitman, 1969; Senior and MacLennan, 1970).

Several lines of evidence from many laboratories indicate that mitochondrial ribosomal RNA and at least some transfer RNA is coded for by mitochondrial DNA. Initially it was shown

that RNA extracted from mitochondrial fractions could hybridize *in vitro* with both mitochondrial and nuclear DNA (Fukuhara 1967b; Wintersberger and Viehhauser 1968; Rabinowitz and Swift, 1970). However, given that the DNA preparations are pure, there are at least two interpretations of this result. It is possible that there is considerable sequence homology between mitochondrial and nuclear DNA. Alternatively, it is possible that the mitochondrial RNA is a mixture (e.g. because of cytoplasmic RNA contamination or because mitochondrial RNA is heterogeneous in origin) so that part hybridizes with nuclear DNA and part with mitochondrial DNA. Fukuhara (1970) has pointed out that the existence of sequence homology can only be demonstrated by showing that the same RNA molecule can hybridize with both DNA species. Using such a technique (hybridization - dehybridization - rehybridization), Fukuhara (1970) has shown that mitochondrial and nuclear DNA have little sequence homology, i.e. the RNA extracted from mitochondrial fractions is heterogeneous, but part of this is coded by mitochondrial DNA (for reviews see Ashwell and Work, 1970; Küntzel, 1971). More recently purified mitochondrial ribosomal RNA has been shown to hybridize with mitochondrial DNA (Borst and Grivell 1971).

The hybridization studies, suggesting that at least part of mitochondrial RNA is synthesised on a mitochondrial DNA template, are supported by inhibitor and labelling studies. Ethidium bromide, which specifically inhibits mitochondrial DNA replication (Zylber, Vesco and Penman, 1969; Goldring, Grossman, Krupnick, Cryer and Marmur, 1970), also inhibits the synthesis of mitochondrial RNA (Attardi, Aloni, Attardi, Lederman, Ojala, Pica-Mattoccia and Storrie, 1971; Yu, in preparation). If mitochondrial RNA is coded by mitochondrial DNA then the number of cistrons remaining for mitochondrial proteins is even more limited. Nevertheless, this RNA synthetic capacity of the mitochondria, which has also been demonstrated by *in vitro* incorporation studies (Ashwell and Work, 1970), represents an important mitochondrial function.

The situation with respect to the capacity of mitochondria to synthesise membrane lipids likewise suggests an extremely restricted degree of autonomy. It is reasonably well established that isolated mitochondria can incorporate acetate into fatty acids, by both *de novo* synthesis and elongation of existing fatty acids (Christ and Hulsmann, 1962; Harlan and Wakil, 1963; Barron, 1966; Mazliak, Stoll, and Abdelkader, 1968) although it is thought that mitochondria cannot desaturate fatty acids (Barron, 1966; Christ, 1968). It has also been shown that mitochondria have an essential role in the conversion of carbohydrate into fatty acids (Watson and Lowenstein, 1970) as a consequence of the mitochondrial localization of pyruvate dehydrogenase. Nevertheless, the major cellular site of fatty acid synthesis is on the microsomal membranes (Bloomfield and Bloch, 1960; Klein, 1965). Likewise, sterol synthesis occurs in the microsomal fraction (Klein, 1965) and there is no evidence to suggest that mitochondria can synthesise sterols.

Many studies with isolated mammalian mitochondria have suggested that these organelles are able to synthesise phospholipid (for example, Bygrave and Kaiser, 1968; Stoffel and Schiefer, 1968) although the major site of phospholipid synthesis is microsomal (Dawson, 1966). More recently McMurray and Dawson (1969) have concluded that mitochondria have at most a very limited ability to synthesise phospholipids such as phosphatidyl choline, and that the earlier reports can be attributed to microsomal contamination. Bygrave and Roberts (1971) have now revised earlier claims that mitochondria could synthesise phosphatidyl choline and phosphatidyl serine, although it appears that phosphatidyl ethanolamine has at least a dual origin in the cell.

These studies suggest that mitochondrial biosynthetic systems can produce only a small portion of the organelle protein and lipid components. Although it is not possible from present evidence to define exactly the nature of the small group of proteins synthesised by the mitochondrial

protein-synthesising system, Ashwell and Work (1970) in a recent review propose that most workers would regard 5 percent of the total mitochondrial protein as the upper limit. The proportion of the total mitochondrial lipids made by mitochondrial lipid-synthesising systems may be even less. If ideas concerning the endosymbiotic origin of mitochondria are correct then mechanisms to explain the loss of mitochondrial autonomy have to be proposed.

(b) Mechanisms of Mitochondrial Biogenesis

The overall picture that emerges from the studies discussed above shows that many biosynthetic systems, in both mitochondrial and cytoplasmic cell compartments, are involved in the biogenesis of mitochondria. This raises intriguing questions concerning the temporal and spatial co-ordination of these biosynthetic systems in the reproduction of mitochondria from generation to generation of cells.

Proposed mechanisms of mitochondrial biogenesis can be grouped under three headings:
(i) formation by differentiation of existing cell membranes (eg. from nuclear, endoplasmic reticulum, or plasma membranes), (ii) *de novo* synthesis, i.e., growth from small, non-functional sub-structures, and (iii) reproduction by division and growth. The first of these mechanisms, differentiation of cell membranes, has depended largely on circumstantial evidence from electron microscopic observation of mitochondrial association with various membranes. The variability of such observations, and their circumstantial nature in view of the fact that mitochondria are apparently quite mobile in the cell, weakens the evidence presented. Also, it is difficult to imagine how mitochondrial DNA could be conserved with such a mechanism. *De novo* synthesis has been considered seriously largely because of a study of the yeast system, and as explained below has now been discounted, at least in its original form. The third postulate, that new

mitochondrial populations arise by growth and division of existing ones, has a much stronger basis. These ideas have been extensively reviewed (for example, Novikoff, 1961; Roodyn and Wilkie, 1968; Ashwell and Work, 1970) although the relative support given by different authors to each point of view varies considerably. However, it should be pointed out that the proposals are not necessarily mutually exclusive.

In considering the biogenesis of mitochondria several limitations must be taken into account:-

(i) the mitochondrial genome can code for only a small fraction of the proteins of the organelle;

(ii) mitochondrial biosynthetic systems can synthesise only a small portion of the mitochondrial components;

(iii) mitochondrial membranes probably consist of sub-units (see later);

(iv) in at least some organisms mitochondrial elements undergo dilution and redistribution in a manner consistent with division of the organelle.

A more detailed elaboration of the 'growth and division' hypothesis, which suggests growth (after division) by accretion of pre-formed sub-units, is necessary. With this in mind the biogenesis of mitochondria in yeast will be described more fully below.

4. CONTROL OF MITOCHONDRIAL BIOGENESIS IN *SACCHAROMYCES*.

(a) Oxygen and mitochondrial development

(i) The effects of anaerobic growth conditions on yeast mitochondria

Perhaps the most important single environmental factor controlling mitochondrial biogenesis and development in species of *Saccharomyces* is the availability of oxygen. The members of this genus are facultative anaerobes. When grown anaerobically these cells have negligible respiration and severely decreased levels of such typical mitochondrial components as cytochromes and respiratory enzymes (Slonimski, 1953). Aeration of these anaerobically-grown yeast results in the rapid development of mitochondrial function (Ephrussi and Slonimski, 1950).

The actual role or roles of oxygen has been extensively debated. It is known that in eucaryotic organisms such as yeast many biosynthetic reactions directly involve oxygen. Among these are reactions in the pathways leading to the synthesis of unsaturated fatty acids, sterol, heme, and quinones (for reviews see Goldfine and Bloch, 1963; Goldfine, 1965). As well there is the important function of oxygen as the terminal electron acceptor in respiration. The relationship of these two important functions of oxygen - in biosynthesis and energy production - to the oxygen-induced formation of mitochondria is not known. It is possible that oxygen is involved at the level of transcription or translation as a regulator, or that the regulator molecule(s) is a metabolite(s) the synthesis of which is determined by the environmental oxygen tension. The importance of oxygen in the regulation of the metabolism of microorganisms is fully discussed in a review by Wimpenny (1969).

Over the last few years considerable effort has been directed towards an understanding of the morphology and biochemistry of anaerobically-grown yeast, particularly of *S. cerevisiae*. Because this yeast requires unsaturated

fatty acid and ergosterol for sustained anaerobic growth (Andreasen and Stier, 1953, 1954), many of the studies on anaerobic growth have been concerned with the effects of lipid supplementation. Consequently the effects of anaerobiosis and lipid supplementation are best considered together.

Wallace and Linnane (1964) and Linnane (1965) reported that cells grown anaerobically without lipid supplements contained no mitochondrial structures detectable by electron microscopy. Linnane and co-workers used this observation to support the postulate that mitochondria are formed *de novo* following aeration of anaerobically-grown yeast. Morpurgo, Serlupi-Crescenzi, Tecce, Valente, and Venettacci (1964) also made the same observation, but found that cells grown under conditions of lipid depletion were considerably damaged. Furthermore, both groups reported that cells grown anaerobically in the presence of ergosterol and unsaturated fatty acids contained "mitochondrial-profiles", i.e. mitochondria lacking cristae (Morpurgo *et al.* 1964; Wallace, Huang, and Linnane, 1968). However Polakis, Bartley and Meek (1964, 1965) did not observe these profiles. It is difficult to compare work from the various laboratories because of differences in growth conditions, but there was general agreement on the lack of "profiles" in cells grown anaerobically without lipids.

In all the above electron microscopic studies, potassium permanganate was used as the fixative, because other fixatives such as glutaraldehyde or osmium tetroxide did not effectively penetrate the intact cell wall (Vitols, North, and Linnane, 1961). However, permanganate is a poor fixative of cytoplasmic structure. For example no ribosomes and few protein structures are preserved by it (Luft, 1956). The other serious problem associated with the use of permanganate is related to its mode of action. Dreher, Schulman, Anderson, and Roels (1967) and Shah (1968) have shown that permanganate reacts only with unsaturated bonds in lipid monolayers, and that preservation of structure is

dependent upon the presence of a sterol such as cholesterol. Korn (1966) has also emphasised the limitations of the use of permanganate. It would be expected on the basis of these results that membranes such as those in anaerobically-grown lipid-depleted cells, which have very low levels of unsaturated fatty acid and sterol (see below), would be particularly susceptible to destruction by permanganate, as well as being only poorly stained.

Because of these limitations in the technique of positive fixation with permanganate, Schatz and co-workers have applied freeze-etching in conjunction with a biochemical study to the examination of anaerobic cells. Using these methods they have found that cells grown anaerobically with or without essential lipid supplementation contain mitochondria-like (promitochondrial) structures (Plattner and Schatz, 1969) as well as oligomycin-sensitive ATPase and mitochondrial DNA (Criddle and Schatz, 1969; Fukuhara, 1967b). These authors conclude that the mitochondria of yeast persist during anaerobic propagation, even though they may be non-functional (at least in terms of oxidative phosphorylation), and in spite of the fact that their enzymic and lipid composition may be radically altered.

Damsky, Nelson and Claude, (1969) have verified the presence of promitochondria in lipid-limited anaerobic yeast. The anaerobic cells were pre-fixed with paraformaldehyde or glutaraldehyde then the cell walls ruptured by shaking with glass beads. The fixation was completed with glutaraldehyde and osmium tetroxide. Watson, Haslam, Veitch, and Linnane (1971), using prefixation of spheroplasts with glutaraldehyde, have also recently described the isolation of promitochondria ('primitive mitochondrial structures') from both lipid-supplemented and lipid-depleted anaerobic cells.

Given that the growth of yeast cells without oxygen (with or without lipid supplementation) does not result in the total loss of mitochondrial structure, the effects of

oxygen deprivation on the biochemical nature of the promitochondrial structures remain largely undefined. The biochemical properties of promitochondria are further discussed in chapter IV. The question that arises from these studies is the relationship of the promitochondria of anaerobic cells to the mitochondria of aerobically-grown yeast, and this is considered below.

(ii) The relationship between promitochondria and mitochondria : the anaerobic to aerobic transition.

Essentially, the delineation of a relationship between promitochondria and mitochondria involves correlation of the biochemical events leading to the development of functional mitochondria with the differentiation of promitochondria. This means that it is necessary to show a precursor-product type of relationship. It is worthwhile considering the events associated with this transition in some detail.

In *S. cerevisiae* certain aspects of this transition have been well-characterized. The early studies of Chin (1950), Ephrussi and Slonimski (1950), and Slonimski (1956), outlined the phenomenon of respiratory adaption in which exposure of anaerobically-grown cells to oxygen induced the development of respiration. This is accompanied by the formation of aerobic cytochomes and respiratory enzymes, both particulate (eg. NADH and succinate dehydrogenase) and soluble (eg. malate dehydrogenase and fumarase). Concomitant with this respiratory adaption is the synthesis of unsaturated fatty acids and ergosterol (Klein, 1955; Kovac, Subik, Russ, and Kollar, 1967) as well as ubiquinone (Sugimura and Rudney, 1960). It also appears that a relatively minor synthesis of mitochondrial DNA takes place on adaption (Mounolou, Jakob, and Slomimski, 1966; Rabinowitz, Getz, Casey, and Swift, 1969). Fukuhara (1967a, b) has described the induction of a certain class of RNA; this is discussed in more detail later. The induction of mitochondrial enzymes probably represents *de novo* protein synthesis, as the induction is sensitive to inhibitors of protein synthesis.

Because the actual mechanism of oxygen-induction is not understood, the interrelationships between the syntheses outlined above are poorly understood. It would be possible to postulate for example that oxygen causes the synthesis of a particular class of DNA and RNA, which is then translated into mitochondrial proteins, but the evidence is largely circumstantial. Another important aspect of this problem is the interrelationship between the lipid and protein syntheses that occur on aeration. This would be especially true if, as appears likely, many of the mitochondrial enzymes are lipoproteins in their active form. It may be, for example, that lipid synthesis and protein synthesis are tightly co-ordinated by feedback controls. Because the anaerobic to aerobic transition involves the synthesis of both lipids and proteins, it is possible that this system would be suitable for an examination of problems related to co-ordination. In addition, because functional mitochondria can be formed at the same time, the transition to aerobic conditions provides an opportunity to study the assembly process, and the relationship between promitochondria and mitochondria. These relationships are considered in detail in later chapters: the problem of the interrelationship between lipid and protein syntheses, and the integration of these elements into functional mitochondria is the central theme of this thesis.

(b) Catabolite Repression

The other major environmental factor which affects the development of yeast mitochondria is catabolite repression. The phenomenon of catabolite repression (Magasanik, 1961) is observed as a reduction in the rate of synthesis of certain enzymes, particularly those concerned with catabolism, in the presence of a readily-metabolized carbon source (Paigen and Williams, 1970). The effects of catabolite repression also result in quite profound modification of cell structure, for example by preventing flagella production in *E. coli*, budding in *Saccharomyces*, and mycelium development in *Mucor*.

In yeast, catabolite repression of respiration by glucose was first established by Ephrussi, Slonimski, Yotsuyanagi, and Tavlitzki (1956). Since then many workers have reported that components of the mitochondrial respiratory chain such as respiratory enzymes and cytochromes are subject to catabolite repression (for example, Strittmatter, 1957; Ohaniance and Chaix, 1964; Polakis, Bartley, and Meek, 1964, 1965; Polakis and Bartley, 1965; Jayaraman, Cotman, Mahler and Sharp, 1966; Somlo, 1968; South and Mahler, 1968). As well, mitochondrial structure and number are strongly affected by catabolite repression (Yotsuyanagi, 1962; Polakis *et al.* 1964; Linnane, 1965; Jayaraman *et al.* 1966); aerobic growth of yeast under glucose repression has been found to lead to a reduction in the number of mitochondria to about 20 per cent of that found in derepressed cells, and the formation of cristae in repressed cells is very restricted.

Some groups have claimed that catabolite repression decreases the amount of mitochondrial DNA (Tewari, Votsch, Mahler, and Mackler, 1966; Moustacchi and Williamson, 1966). More recently Fukuhara (1969) has reported that repression (or anaerobiosis) does not affect the relative amount of mitochondrial DNA, and that the earlier findings were due to faulty technique. Claims that catabolite repression affects lipid synthesis and the lipid composition of mitochondria (Lukins, Jollow, Wallace, and Linnane, 1968) are discussed later.

The extent of catabolite repression is known to depend on the concentration and type of carbon source in the medium. In general, substrates that are most readily fermented lead to the greatest repression (for review see Paigen and Williams, 1970). Thus glucose, which is rapidly fermented, causes severe repression. The degree of repression of cells grown for the same period is proportional to the initial concentration of glucose in the medium (Strittmatter, 1957; Polakis *et al.* 1964). Galactose, which has a slower rate of fermentation than glucose, causes less repression

(Strittmatter, 1957; Polakis *et al.* 1965) and sugars such as mellibiose and raffinose, which are very slowly fermented, cause little repression. Galactose appears to offer a good compromise between growth rate and degree of repression, and has been used as the carbon source in experimental studies described later in this thesis.

Questions relating to the nature of the effector molecule(s), the concentration of which determine the extent of repression, and the mechanism of repression, i.e. whether the effector molecule(s) act to control transcription or translation, or both, are largely undecided. Many theories have been proposed, but as these have been extensively reviewed (Magasanik, 1961; Paigen and Williams, 1970) they are not discussed further.

(c) The genetic control of yeast mitochondria

The susceptibility of microorganisms to genetic analysis is one of their main advantages as an experimental system. This is particularly so for mitochondrial studies in *S. cerevisiae*, as mutational changes that result in a non-functional respiratory system do not affect the viability of these facultative anaerobes, whereas similar mutations may be lethal to more advanced organisms. Probably the most studied mutation affecting mitochondria in yeast is 'petite' (denoted ρ^-), so called because it results in the formation of small colonies on solid media containing low concentrations of fermentable substrate (Ephrussi, 1953; see Nagai, Yanagishima, and Nagai, 1961, for review of earlier work).

Two types of cytoplasmic (non-Mendelian) petites are known. One is the neutral petite which, when crossed with a wild-type cell, gives a diploid zygote that on sporulation yields ascospores which all form normal colonies, i.e., give a 0 - 4 segregation, cf. a 2:2 segregation for nuclear (Medelian) genes. The other type is the suppressive petite, in which a proportion of the diploid cells, derived by vegetative growth from the diploid zygote formed in a cross

with the wild-type, are petite (ρ^-). These results have been interpreted to mean that replication of the factor, ρ, (a postulated element that is, or controls replication of, the mitochondrial genome) is under control of a suppressive factor, present in suppressive strains (Rooodyn and Wilkie, 1968). Mitochondrial formation is also under control of nuclear genes.

Earlier workers found that petite mutants were respiration-deficient, and lacked cytochromes a and b. Even though the petite was able to show some adaptive response to oxygen after anaerobic growth. Slonimski (1956) and Chantrenne (1958) showed that cytochrome c and catalase, both of which are absent from anaerobically-grown petite cells, are formed on aeration.

Many agents have been found to induce the petite mutation, for example acridine dyes such as euflavine and proflavine, heavy metals such as copper, nickel and manganese, some nitrophenols, UV irradiation, and heat (Nagai *et al.* 1961). The mode of action of these inducers remains unknown, although it is known that at least some of the acridine dyes interact with nucleic acids, so the inference is that nucleic acid function is disturbed in some way.

By 1964 when it was firmly established that plant plastids contain a characteristic species of DNA, the evidence for DNA in mitochondria depended on histochemical and electron microscopic studies (for review of this work see Gibor and Granick, 1964) as well as the genetic data. It was claimed that the cytoplasmically-inherited petite mutation led to changed mitochondrial morphology (Yotsuyanagi, 1962). The inference drawn was that mitochondrial DNA is the site of the genetic change, but as discussed later, the early electron microscope studies of petite mitochondria were to some extent misleading.

More recently, several groups have compared the mitochondrial DNA of wild-type and petite yeast in biochemical studies. Although there is disagreement about the extent of change, it has been generally found that the

petite mutation causes alteration of the bouyant density of mitochondrial DNA (see Roodyn and Wilkie, 1968, and Carnevali, Morpurgo, and Tecce, 1969, for a discussion of this question), and that the alteration may be extensive; this is consistent with the known lack of reversibility of cytoplasmic petites. The implication from these studies is that mitochondrial DNA is the rho factor.

Recent work of Goldring, Grossman, Krupnick, Cryer, and Marmur (1970) and Goldring, Grossman and Marmur (1971) supports this conclusion. These workers examined the effect of ethidium bromide, a potent petite-forming mutagen in *Saccharomyces*, on the synthesis of mitochondrial DNA in yeast. They found that this drug selectively inhibits mitochondrial DNA synthesis in *S. cerevisiae*, with the result that dividing cells subjected to prolonged treatment contain no mitochondrial DNA, while shorter treatments resulted in petites with mitochondrial DNA of a reduced size : the rate of alteration of the mitochondrial DNA could be correlated with the rate of petite formation. Similar results have been reported by Nagley and Linnane (1970), and Nagley, Gingold, Lukins and Linnane (1971).

It would be expected that such gross alterations to the structure or amount of mitochondrial DNA during petite induction should have quite drastic effects on the formation of mitochondria, if this DNA is active as a template. The fact that respiration-deficient mitochondria result indicates that mitochondrial DNA is an important determinant of mitochondrial development. However, as indicated earlier, it appears that relatively few mitochondrial components are made by the mitochondrial protein-synthesising system. If what is synthesised by the mitochondrial protein-synthesising system is coded for by mitochondrial DNA then this DNA contributes quantitatively little information to the biogenesis of mitochondria : this is supported by calculations of the coding capacity of the mitochondrial DNA, as discussed above.

Several postulates relating to the role of mitochondrial DNA may be considered. It is possible that the few proteins that mitochondrial DNA may code for act as 'basic components' the synthesis of which is a prerequisite for the extra-mitochondrial synthesis and assembly of other mitochondrial components. A structural element(s) of organelle membrane could be considered in this respect. Alternatively, it is possible that mitochondrial DNA codes for the synthesis of 'effector' proteins which control the synthesis of mitochondrial proteins on mitochondrial and/or cytoplasmic ribosomes. In this case the structural genes for mitochondrial components would be located in the nuclear chromosomes and the mitochondrial genome would contain the regulator genes. As the synthesis of different classes of mitochondrial proteins are differently regulated, as described later, it may be that both of these mechanisms, or variations of them, operate.

Whatever the case, it is apparent that mitochondrial DNA has an important function in the control of mitochondrial biogenesis, even though its coding capacity is limited. This is further considered in a later chapter along with the biochemical properties of petite mitochondria.

5. THE STUDY OF THE MITOCHONDRION AS A MODEL MEMBRANE SYSTEM.

(a) The structure of membranes

Because mitochondrial biogenesis involves the synthesis and assembly of a functional membrane system, and because studies of the structure and function of mitochondria have been more extensive than of other membrane systems, some workers have attempted to generalize to other cell membranes (for example, Green and Perdue, 1966; Green and Tzagoloff, 1966). Hence over the past few years the mitochondrion has increasingly come to be considered as a model membrane system. It is therefore worth considering the proposed structure of the mitochondrial membrane, and to compare it with other postulated membrane structures.

From extensive studies on the composition and structure of mitochondrial membranes, Green and co-workers (for review see Green and Perdue, 1966; Green and Tzagoloff, 1966) have proposed that membrane structure depends on the association of globular lipoprotein subunits. This proposal was founded on studies of the four complexes of the electron-transfer system of beef heart mitochondria in which it was shown that these could be dissociated, with loss of activity, then reaggregated with restoration of activity. In these studies attempts were made to relate the morphology of the mitochondrial membranes, as viewed by electron microscopy, to the enzymology and function of the membranes. As well, Green and co-workers claimed that the lipid-protein bonding in membranes was hydrophobic, in contrast to other proposals described below. Evidence obtained from studies with chloroplasts has also led to the proposal that membranes of these organelles consist of hydrophobically-bonded subunits (Benson, 1966; Benson, Gee, Ji, and Bowes, 1971).

In support of the subunit model, reversible disaggregation has been reported with membranes of *Mycoplasma laidlawii* (Razin, Morowitz, and Terry, 1965) and *Halobacterium halobium* (Brown, 1964, 1965), although more recent evidence has thrown some doubt on these reports (Engelman, Terry, and Morowitz, 1967).

Classical ideas on the structure of membranes stem largely from the work of Gorter and Grendel (1925). These workers extracted lipids from erythrocyte membranes and measured the surface area of derived lipid monolayers. They found that the extracted lipids provided enough material to cover the cells twice with a molecular monolayer. They suggested that the cell membrane was a bimolecular phospholipid leaflet with the hydrophobic hydrocarbon chains opposing each other and the polar portions of the molecules on the outside of the leaflet. Danielli and Davson (1935) proposed a model in which the bimolecular lipid leaflet was given a protein coat. Robertson (1957), reviewing additional data from several laboratories, modified this

model and postulated that the protein layers were in the extended β - form, rather than in the globular form, and that one side of the membrane may contain mucoprotein and so be more hydrophilic; he called this structure the unit membrane model, and suggested that it might be the basic structure of all biological membranes.

Two important features of the unit membrane model, namely, the continuous lipid leaflet oriented with apolar 'tails' inwards and the protein held to the leaflet via polar interactions, have been the centre of most of the recent debate on membrane structure. Evidence for and against the unit membrane and the sub-unit models has recently been thoroughly reviewed (Hendler, 1971). The general conclusion from this review was that while the unit membrane model needs some modification, it is supported by most of the evidence, particularly that obtained since 1966. The most recent evidence form X-ray diffraction studies of emulsions of isolated membranes of *Mycoplasma laidlawii*, erythrocytes, and nerve endings (Engelman, 1970; Wilkins, Blaurock, and Engelman, 1971) and the comparison of these with X-ray diffraction patterns of artificial lipid bilayers (Levine and Wilkins, 1971) has confirmed that biological membranes have large areas of lipid bilayer structure. It is important that the earlier X-ray studies on myelin have been extended to other membranes, as many authors have considered myelin to be a non-representative membrane. It is worth noting that no such X-ray study has been carried out on mitochondrial membranes.

If the unit membrane model is basically correct, modifications that seem necessary (Hendler, 1971) have to allow for considerable α-helicity in the membrane proteins : it is possible that different conformations of membrane protein explain the different membrane thicknesses observed (75 ± 25 Å). As well, the model should allow for regions of hydrophobic bonding between membrane lipids and proteins, and possibly for regions of exposed lipid to explain the susceptibility to lipases and the interaction of membrane with lipid-specific antibodies.

These considerations take into account some of the objections raised by Green and others (for example Korn, 1966) and seem to at least partly remove the necessity of postulating alternate basic structures. However it is possible to take the converse approach and modify the subunit model to incorporate the strong evidence for large areas of lipid bilayer structure in membranes. Recently, Green and his collaborators have done just this, and in a modified version of the subunit model they propose that the membrane continuum is made up of a double layer of globular protein molecules (cf. earlier lipoprotein subunits) interspersed with a lipid bilayer (Vanderkooi and Green, 1970; Green, Korman, Vanderkooi, Wakabayashi, and Valdivia, 1971). The result is a sparse bilayer with a non-polar interior and a polar exterior (Vanderkooi and Green, 1970).

It appears then that the unit membrane and the subunit membrane models are in some respects becoming quite similar. The controversy remaining may be resolved by a precise estimation of the percentage of lipid bilayer structure and a comparison of this with that predicted by the two models. Nevertheless, we are still far from a complete explanation of the extreme selectivity of membranes and of their biological diversity in terms of structure.

(b) Lipid and protein components of membranes

(i) Structural proteins of mitochondria.

Relatively little is known about mitochondrial membrane proteins, and this uncertainty has been brought into sharp relief by several recent papers that question the whole basis of the earlier studies that led to the development of the 'structural' protein concept. Even so, the studies on 'structural' proteins have been the source of many ideas concerning the organisation and function of mitochondrial membrane proteins, so they are worth consideration. As it has turned out, 'structural' protein and ATPase are closely related, so in this section they are considered together.

The ATPase activity associated with the inner mitochondrial membrane as an essential part of the oxidative phosphorylation system has been the focus of much experimentation and speculation. The work of Racker and collaborators (for example, Racker, Tyler, Estabrook, Conover, Parsons, and Chance, 1965; Racker and Bruni, 1968) has established that ATPase activity in beef heart mitochondria can be correlated with the projecting headpieces of the inner membrane seen when these membranes are viewed in the electron microscope after negative staining. (This is to be contrasted with the work of Mitchell, 1968, in which it is postulated that ATPase is an integral part of the inner membrane). Two components of the ATPase complex have been examined in most detail. The first of these is the soluble enzyme (denoted F_1) which is activated by Mg^{++}, is cold-labile (due to subunit dissociation), and is insensitive to oligomycin. When F_1 is complexed in the mitochondrial membrane it is cold-stable and oligomycin-sensitive, and it is postulated that this is due to interaction with a second component(s), a factor conferring oligomycin-sensitivity (Kagawa and Racker, 1966a, b; Racker and Bruni, 1968). The subunit structure of F_1 has been the source of some disagreement. Penefsky and Warner (1965) suggested that F_1 was a complex of about 10 homogeneous subunits of approximately 29,000 mol. wt. Other studies (summarized by Senior and MacLennan, 1970) have found that ATPase complexes are heterogeneous, consisting of at least four different components. Senior and MacLennan (1970) have recently isolated what they call a major component of F_1, with a molecular weight of 80,000 and which appears to be very stable. In contrast, Forrest and Edelstein (1970) propose a hexameric structure for ATPase, with subunits of 46,000 mol. wt.

In yeast recent studies have concentrated on the biosynthesis of ATPase, and it has been shown that F_1 activity is affected by the growth conditions in a way that is similar to other inner-membrane components (Somlo, 1968; Schatz, 1968). Schatz (1963, 1965) showed that ATPase activity was relatively low in anaerobically-grown cells; this activity

increased on aeration (Somlo, 1968). ATPase is also subject to glucose repression. Tzagoloff (1969a, b) demonstrated that ATPase activity increased on derepression in aerobic culture, and that this increase was due to a net synthesis of F_1. This derepression system was used (Tzagoloff, 1969b) in a study of the biosynthesis of the ATPase complex : he concluded that F_1 was synthesised on the cytoplasmic ribosomes (cf. Haldar, Freeman and Work, 1966). This conclusion is supported by studies on the ATPase of petite mutants (Schatz, 1968; Kovac and Weissova, 1968). Petite mutants contain ATPase activity, but it is oligomycin-insensitive. This result has been taken to indicate a selective loss of the component donating sensitivity on mutation to the petite, implying that the different components of ATPase are under separate genetic control. The biosynthesis of ATPase is considered further in a later chapter.

The concept of a mitochondrial 'structural' protein has been examined in many recent reviews (Rothfield and Finkelstein, 1968; Ashwell and Work, 1970; Senior and MacLennan, 1970) and is here discussed only in outline. Early workers (Criddle, Bock, Green, and Tisdale, 1962; Richardson, Hultin, and Fleischer, 1964) isolated an insoluble protein fraction from beef heart mitochondria. It was claimed that this 'structural' protein formed the "backbone" of mitochondrial membranes. This protein was thought to be homogeneous, and accounted for almost half of the total mitochondrial protein. In addition, the protein was able to bind respiratory chain components (Criddle *et al.* 1962) and phospholipids (Richardson *et al.* 1964). Woodward and Munkres (1966a, b, 1967) prepared a similar 'homogeneous' structural protein from *Neurospora* not only from mitochondria but also from other cell fractions, including the cytosol fraction. This structural protein from *Neurospora* bound malate dehydrogenase, while mutant structural proteins had altered binding properties.

The claim that these preparations were homogeneous has been questioned by several groups, including those that made the original claim (Lenaz, Haard, Lauwers, Allman,

and Green, 1968; Green, Haard, Lenaz, and Silman, 1968). On the basis of these more recent studies, the concept of a 'structural' protein was first broadened and then further changed (Green *et al.* 1968) because what was previously called structural protein was thought to have been isolated from detachable structures on the surface of the membrane : a new non-catalytic component of membranes, 'core' protein, was introduced.

The concept that 'structural' protein is attached to membranes, and is not an integral part of them, puts one in mind of the observations discussed above on the properties of mitochondrial ATPase and suggests a relationship between the two. Two recent papers add strong support to this relationship. Schatz and Saltzgaber (1969b) added labelled F_1 to ATPase-deficient sub-mitochondrial particles. They then prepared 'structural' protein from the reconstituted particles, and found that most of the radioactivity was recovered in this fraction, although the specific radioactivity was lower than that of the originally added F_1. Senior and MacLennan (1970) developed a new method for the preparation of 'structural' protein so that it was soluble in dilute aqueous buffer. They showed that this preparation was heterogeneous on polyacrylamide gels, and identified a major component of the structural protein as the major component of F_1. These workers concluded that 'structural' protein preparations "are not relevant to a study of mitochondrial membrane proteins or organiser proteins because they contain denatured enzymic and non-membrane protein in high quantity". They added that the concept of 'core' protein was not invalidated by their work, but that there is no evidence for it : at the moment this concept has only heuristic value.

As discussed in an earlier section, there is considerable evidence that 'insoluble' mitochondrial protein is the product of the mitochondrial protein-synthesising system, and it has been suggested that 'insoluble' protein is the 'structural' protein of the inner membrane (Beattie, 1969b). Because of the above discussion this view now would need to be treated with caution.

(ii) 'Structural' lipid components of membranes.

Studies on membrane composition have been limited by the availability of pure membrane preparations, and most data have been obtained using erythrocyte or nerve (myelin) membranes as representative of mammalian systems, and *Mycoplasma* and bacterial membranes representing those of microorganisms. More recently Pfleger, Anderson and Snyder (1968) have described the preparation and lipid composition of purified plasma membranes from rat liver cells using an isolation method involving zonal centrifugation. Purification problems also apply to mitochondrial preparations : firstly because of the heterogeneity of the membranes of the organelle itself (inner, outer, and cristal membranes),and secondly because of contamination with microsomal membrane. This is true even in yeast where the endoplasmic reticulum appears to be poorly-developed in comparison with that of mammalian cells (Klein, Volkman, and Weibel, 1967). Yeasts have the additional disadvantage (as experimental systems) of a relatively thick cell wall, which makes the preparation of membranes very difficult as quite drastic cell breakage methods are usually necessary.

The membrane models discussed earlier assign a key role to lipids, particularly phospholipids,and this has been supported by chemical analysis of many cell membranes. The lipid composition of membranes has been the subject of many recent reviews (Green and Tzagoloff, 1966; Rothfield and Finkelstein, 1968; Van Deenen, 1970). Only those questions immediately relevant to the topic of this thesis are considered here.

It appears that all cell membranes contain phospholipids, although the types and relative proportions differ considerably. For example phosphatidyl choline is the major phospholipid in most eucaryotes, while it is only present in a few bacteria; bacteria generally have phosphatidyl ethanolamine as the major membrane phospholipid (Asselineau, 1966). The phospholipid content of whole yeast cells (Letters, 1966: Jollow, Kellerman and Linnane, 1968; Paltauf and Schatz,

1969) and yeast mitochondria (Jakovcic, Getz, Rabinowitz, Jakob, and Swift, 1971; Lukins *et al.* 1968) are remarkably similar, and both are similar to that of mitochondria from mammalian tissues (Fleischer, Fleischer, Rouser, Casu, and Kritchevsky, 1967; Getz, Bartley, Lurie, and Notton, 1968). Cardiolipin, or diphosphatidyl glycerol, appears to be an extreme example of phospholipid specialization as it has only been found in mitochondrial membranes (Green and Tzagoloff, 1966; Getz *et al.* 1968; Jakovcic *et al.* 1971). Furthermore, cardiolipin is located only in the inner mitochondrial membrane (Parsons, Williams, Thompson, Wilson and Chance, 1967; Stoffel and Schiefer, 1968; McMurray and Dawson, 1969). Very recently Jakovcic *et al*, (1971) have concluded that the content of yeast cardiolipin is correlated with, and is a good indicator of, the degree of mitochondrial membrane development. This phospholipid is not necessarily associated with respiratory development as petite yeast and anaerobically-grown wild-type yeast still contain cardiolipin. Van Deenen (1970) has emphasised another type of phospholipid variation. The lecithin of mammalian membranes appears to consist of at least 20 molecular species in which the esterified fatty acids differ in type and position, and the relative proportion of these species differs from tissue to tissue within the same animal. This implies an environmental control of the lecithin composition, as all the tissues of one animal have the same genetic complement.

The other major membrane lipid component found in almost all organisms is sterol; a notable exception is the eubacteria (Asselineau and Lederer, 1960). Little is known about the role of sterols in cell membranes. Studies with artificial membrane systems (Dreher *et al.* 1967; Ladbrooke, Williams, and Chapman, 1968) have shown that sterols are important in maintaining the stability of these membranes. Ladbrooke *et al.* (1968) have further suggested that sterol may be able to control the fluidity of the hydrocarbon chains, giving stability over a wide temperature range.

In yeasts of the genus *Saccharomyces* ergosterol is the major sterol (Dulaney, Stapley, and Simpf, 1954), cf. cholesterol in mammals. Under anaerobic conditions *Saccharomyces* is unable to synthesise ergosterol, and so this sterol becomes a growth factor (Andreasen and Stier, 1954). However the specific requirement for sterol appears to be very flexible. Proudlock, Wheeldon, Jollow, and Linnane, (1968) have shown that the steroid molecule must be planar, have a long alkyl side-chain at C-17, and have a C-3 hydroxyl group. These workers have suggested that sterol has a structural role in membranes, in agreement with the postulate of Ladbrooke *et al*. (1968) discussed above.

Prolonged growth of *S. cerevisiae* under anaerobic conditions also requires an exogenous source of unsaturated fatty acid (Andreasen and Stier, 1953). Like ergosterol, the unsaturated fatty acid requirement is satisfied by endogenous synthesis in the presence of oxygen. After anaerobic growth in the absence of added lipids, the yeast cell levels of ergosterol and unsaturated fatty acid are severely depleted (Meyer, Light, and Bloch, 1963). In these cells the small amounts of unsaturated fatty acid remaining are concentrated in the phospholipid fraction (Jollow *et al*. 1968). It is likely that the unsaturated fatty acid growth requirement exists because the phospholipid components of cell membranes require at least a small amount to maintain membrane structure. (This has not been considered in any membrane model). The functional and/or structural differences between yeast membranes with a high sterol and high unsaturated fatty acid content as occurs in aerobically-grown yeast, and those with a low content of these lipids (in anaerobic, lipid-depleted yeast) are not understood. The fact, however, that yeast cells can remain viable under both conditions again suggests a very great degree of flexibility and tolerance to variation in membrane composition in this organism.

In later chapters the interrelationship between protein and lipid syntheses during the formation of functional

mitochondria is considered. For this study the lipids that have been examined in most detail have been fatty acids and sterol, since they are essential lipids and they represent quantitatively major components of membranes of aerobically-grown yeast cells. Their use as markers of membrane lipid is enhanced by the ease with which they can be measured in different types of preparations, and because they are sensitive indicators of changes that occur in the structure and function of membranes of this organism as a response to alterations in the culture conditions. Another lipid, ubiquinone, with properties strikingly different from those of the lipids mentioned above, has also been examined in some detail in the present study. Because its function appears to be quite different from that of the 'structural' lipids it has been considered separately.

(iii) The function and biosynthesis of Ubiquinone.

The role of ubiquinone in electron transport. The early finding that the loss of succinoxidase activity of beef heart mitochondria following heptane extraction (Crane, Hatefi, Lester, and Widmer, 1957) could be restored with a substance from the heptane extract named coenzyme Q (Lester, Crane, and Hatefi, 1958) or ubiquinone (Morton, Wilson, Lowe, and Leat, 1957) prompted much experimentation as to the role of this important component of electron transport in mitochondria. Ubiquinone is present in almost all organisms, and is localized in the inner mitochondrial membrane of eucaryotes (Ernster and Kuylenstierna, 1969). Current literature contains all possible opinions concerning the importance of ubiquinone, particularly with regard to its proposed function in the respiratory chain. These opinions range from the obligatory presence of ubiquinone in the respiratory chain to its complete absence; even where it is agreed that ubiquinone functions in the pathway there is disagreement as to its position in the chain, in particular whether it is on the substrate side or the oxygen side of cytochrome b (Ernster, Lee, Norling, and Persson, 1969, and references contained therein). Part of the controversy comes

from kinetic studies, in which some workers have claimed that the rate of oxidation/reduction of ubiquinone is too slow to be compatible with the overall rate of electron transport, suggesting that it is not in the mainstream of electron flow. More recent studies have questioned this claim (Ernster *et al*, 1969; Cox, Newton, Gibson, Snoswell, and Hamilton, 1970).

Strong evidence for the participation of ubiquinone in electron transport has been provided by the work of Crane *et al.* (1957), Lester and Fleischer (1959), and Szarkowska (1966). These workers have shown that activity of the succinate and NADH oxidase systems lost following selective lipid extraction could be restored by the specific addition of ubiquinone. Ernster *et al.* (1969) confirmed these findings, and extended them to an examination of the reduction of cytochromes b, c_1 and a by NADH and succinate in submitochondrial particles. They concluded that the succinate and NADH oxidase systems, and their interaction, are dependent on ubiquinone and that ubiquinone is located on the substrate side of cytochrome b. Also these workers state that their findings are in accordance with the concept that ubiquinone functions as a 'mobile' electron carrier (Green *et al.* 1965), acting as a 'homogeneous hydrogen-collecting pool' (Kröger and Klingenberg, 1967) connecting various flavoproteins and the cytochrome system. In bacteria, the position of ubiquinone in the respiratory chain appears to be similar to that proposed by Ernster *et al.* (1969) above for mammalian mitochondria. For example, Jones, (1967) has proposed that ubiquinone functions between flavin and cytochrome b_1, and Cox *et al.* (1970) have suggested a scheme in which the quinone functions both before and after cytochrome b_1.

The biosynthetic pathway for ubiquinone synthesis. It is only very recently that a pathway for ubiquinone biosynthesis has been established with any certainty. Labelling studies had shown that the O-methyl groups of ubiquinone arise from the methyl group of methionine and that the polyprenyl side chain is synthesised via the mevalonate pathway. In addition it was proposed that the benzoquinone

ring is derived in microorganisms from the shikimic acid pathway for aromatic acid biosynthesis (for review of early work see Rudney and Raman, 1966). The discovery that 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid were converted with high efficiency into the benzoquinone ring (Rudney and Parson, 1963) allowed more direct labelling studies to be carried out : these led to the discovery of another key intermediate which accumulates in *Rhodospirillum rubrum* in the dark under nitrogen, and which is converted to ubiquinone when light is admitted. This intermediate was found to be 2-decaprenylphenol (Olsen, Daves, Moore, Folkers, Parson, and Rudney, 1966).

On the basis of the above and similar work, a pathway for the biosynthesis of ubiquinone was postulated (Rudney and Raman, 1966; Friis, Daves, and Folkers, 1966) even though most of the intermediates had not been isolated. More recent evidence raised the question of alternate pathways as Friis, Nilsson, Daves, and Folkers (1967) found a compound that they thought to be a ubiquinone precursor, but which did not fit the proposed pathway. Furthermore, Whistance, Field, and Threlfall (1971) and Spiller, Threlfall, and Whistance (1968) have reported that they were unable to detect multiprenyl phenols in animal or fungal tissues, throwing doubt on the generality of the earlier finding.

Gibson and co-workers have used a different approach that has proved much more successful. Their study has involved the isolation of a series of ubiquinone-deficient mutants of *E. coli* and an examination of these for accumulated intermediates (Cox, Snoswell, and Gibson, 1968; Cox, Young, McCann, and Gibson, 1969). Recently this group has achieved the isolation of all the postulated intermediates of the ubiquinone pathway, providing direct evidence for the existence of the pathway, as well as presenting a mapping of the genes involved (Young, McCann, Stroobant, and Gibson, 1971 a; Young, Stroobant, Cox, McCann, Lawrence and Gibson, 1971b).

Work on the enzymology of the ubiquinone pathway has only been partially successful, and no cell-free system has been found to synthesise ubiquinone. Raman, Rudney, and Buzzelli (1969) reported the conversion of 4-hydroxybenzoic acid into a compound tentatively identified as 3-octaprenyl-4-hydroxybenzoic acid by a cell extract of *R. rubrum*, but they found it was necessary to add an extract from *Micrococcus lysodeikticus*. Their earlier claims of cell-free synthesis of ubiquinone (Raman and Rudney, 1966) were thought to be due to unbroken cells in the extract. Young *et al.* (1971b) have reported that the first enzymic step in ubiquinone biosynthesis from chorismic acid appears to be in the 'soluble' cell fraction, and that the remaining steps appear to be membrane-bound.

The studies discussed above have been concerned with defining the function of ubiquinone and the nature of its biosynthetic pathway. It now appears reasonably certain that ubiquinone is specifically localized in the mitochondrion (like cardiolipin) and functions as an obligatory component of the electron transport system, although the biosynthetic pathway has only been well-defined in *E. coli*. The studies on ubiquinone presented in this thesis have been mainly concerned with the control of ubiquinone biosynthesis; it was thought that a knowledge of this control would provide some insight into the synthesis of a non-protein component of the electron transport system, i.e. ubiquinone may be a good non-protein mitochondrial marker. It was hoped that knowledge of the localization and control of the ubiquinone pathway would be useful in understanding the processes involved in the integration of this non-protein with other components to form mitochondrial membranes. These aspects are discussed further in chapter III.

6. MEMBRANES AND PROTEIN SYNTHESIS

The importance of membranes in biological systems cannot be overstated. Apart from the obvious mechanical property of containment, and the properties of selective permeability and active transport, it now appears that membranes are the sites for many biosynthetic activities. In bacteria, for example, systems for electron transport, lipid synthesis (Carter, 1968) and cell wall synthesis (Pardee, 1968; for review see Rothfield and Finkelstein, 1968) have a membrane location. In eucaryotic organisms the importance of membrane systems is even more evident, as many biological activities are compartmentalized within specific membrane systems.

There has been much interest recently in the relationship between membranes and protein synthesis. Since the studies on liver and pancreas cells by Palade and Siekevitz (1956,a, b) the observation that there are two populations of cytoplasmic ribosomes in mammalian cells has received much experimental support. More recently it has been claimed that free and bound ribosomes are functionally different (Takagi, Tanaka, and Ogata, 1970, and references therein). For example, it has been shown that serum proteins and glycoproteins, i.e. proteins for 'export', are selectively synthesised on membrane-bound polysomes. However, other proteins such as catalase (Takagi *et al.* 1970) and NADPH-cytochrome c reductase, a membrane-bound protein (Campbell, 1970), seem to be synthesised by both classes of ribosomes.

In procaryotic organisms no such definition of ribosome classes has been made. Nevertheless many authors have found that in bacteria most of the ribosomes are membrane-bound, and it appears that membranes may be the major sites of protein synthesis (for reviews see Hendler, 1965; Cundliffe, 1970).

Given that the attachment of ribosomes to membranes has functional significance, the nature of the attachment then

becomes an important consideration, as this may serve as a control mechanism. This is particularly well illustrated by the process of protein secretion in liver cells. The first step is believed to be transfer of the newly-synthesised secretory polypeptides to the cavity of the endoplasmic reticulum. The site of synthesis appears to be membrane-bound ribosomes with the ribosomes attached to the membrane via the 50S subunit (Sabatini, Tashiro, and Palade, 1966) although the mechanism of attachment is uncertain (Campbell, 1970). More recently a model has been proposed (Blobel and Sabatini, 1970; Sabatini and Blobel, 1970) in which the nascent secretory polypeptide grows in an interior space of the large ribosomal subunit, and is then transferred through a discontinuity in the membrane into the membrane cavity.

In bacteria, much evidence has accumulated to support the postulate (Jacob, Brenner, and Cuzin, 1963) that DNA replication requires attachment to the cell membrane (Rothfield and Finkelstein, 1968). In view of the idea that polysomes are formed in bacteria in association with DNA, i.e. while transcription is in progress (Stent, 1966; Schaechter and McQuillan, 1966), the possibility arises that membrane-ribosome association is DNA-mediated. Recent experiments (Cundliffe, 1970) have suggested that this binding mechanism does operate, as well as a direct binding of the ribosomes to the membranes.

The above discussion shows that there is a large body of evidence from bacterial and mammalian systems indicating the importance of membrane-ribosome interaction. With this in mind it might be expected that changes in the composition and/or structure of the membrane would cause a changed membrane-ribosome affinity with consequent effects on protein synthesis. Already there is some evidence to support this idea (Hendler, 1965) and this aspect is further considered in Chapter IV.

CHAPTER II

METHODS

1. ORGANISMS

A wild-type diploid strain of *Saccharomyces cerevisiae* Hansen was used in most experiments. Where noted a cytoplasmic petite isolated from the wild-type was used. In some experiments a haploid mutant (KD115) was used. This mutant is prototrophic in aerobic as well as anaerobic cultures for unsaturated fatty acid. The strain KD115 was a generous gift from Drs Mortimer and Resnick, University of California, Berkeley.

2. CULTURE CONDITIONS

(a) Composition of media

Strains were maintained on agar slopes of a yeast extract-salts medium (Slonimski, 1953). For respiratory-deficient strains this medium contained glucose as carbon source; for the wild-type it contained glycerol. Media for maintenance of KD115 contained Tween 80 (5ml/l) as a source of unsaturated fatty acids.

The liquid growth medium was constituted as follows (per cent, w/v); yeast extract (0.5), ammonium sulphate (0.12), calcium chloride (0.01), sodium chloride (0.05), potassium dihydrogen phosphate (0.1), magnesium sulphate (0.2), ferric chloride (0.0003) and galactose (2.5 for aerobic growth, 4.0 for anaerobic growth). Where indicated this growth medium was supplemented with Tween 80 (0.5 per cent), a water soluble source of unsaturated fatty acid, and ergosterol (0.002 per cent). Strain KD115 was grown with glucose instead of galactose as carbon source, as this strain grows poorly on galactose anaerobically.

In some experiments a synthetic medium was used. The composition of this was (per cent, w/v); KH_2PO_4 (0.4), K_2HPO_4 (0.05), $(NH_4)_2SO_4$ (0.15), NaCl (0.01), $CaCl_2 . 2H_2O$ (0.01), Mg $SO_4 . 7H_2O$ (0.05), ferric citrate, 2 mmole, solution A, 5 ml, and solution B, 1 ml. Solution A contained (mg/l); $CuSO_4.5H_2O$ (40), KI (100), $ZnSO_4.7H_2O$ (100), $Na_2MoO_4.2H_2O$ (100), $Na_2 B_4O_7 .10H_2O$ (100) and $MnSO_4.4H_2O$ (200). Solution B contained (mg/l); calcium pantothenate (100), thiamine (100), inositol (200), pyridoxine hydrochloride (100), nicotinic acid (50), and biotin (20).

Cells grown anaerobically without added Tween 80 and ergosterol are referred to as lipid-depleted; cells grown with these supplements are denoted lipid-supplemented. Media used for aerobic cultures and for aeration did not normally contain the lipid supplements.

(b) Batch culture

Aerobic cultures were grown as shake cultures on a rotary shaker operated at 300 cycles/min. The flasks used contained media to not more than one quarter the volume of the flasks. Anaerobic cultures were grown in sealed flasks filled with media to approximately four-fifths the flask volume. Anaerobic conditions in the cultures were established after inoculation by gassing for 30 min with commercial oxygen-free nitrogen passed through a vanadium sulphate - zinc amalgam train (Meites and Meites, 1948) to remove residual oxygen. The growth vessels were sealed with paraffin : gas evolved during growth was passed off through a water trap and cells were maintained in suspension with a magnetic stirrer.

Aerobic cultures were inoculated with cells grown aerobically to late log phase : initial cell density was 0.001 - 0.002 mg dry wt/ml. Anaerobic cultures were also inoculated with aerobically-grown cells, but to an initial cell density of 0.03 mg dry wt/ml. Substitution of

anaerobically - grown cells for aerobically - grown cells in the inoculum was without significant effect on either the lipid content or the trace amount of respiration found in lipid-depleted cultures after anaerobic growth. However, using anaerobic cells (lipid-depleted) as inoculum resulted in a considerably lower cell yield in stationary phase of lipid-depleted cultures than when aerobically-grown cells were used. This is due to the contribution of essential lipids from the aerobic cells to the total essential lipids available in the medium. These effects on the growth physiology are discussed further in chapter IV.

Unless otherwise noted, cultures were grown at 29° for 16-18 hrs (aerobically) or 20-22 hrs (anaerobically). Cells were harvested by centrifugation. Anaerobic cultures were poured onto crushed ice immediately after opening the anaerobic growth vessel , then harvested. In some cases cycloheximide (final concentration 20 μM) was added in oxygen-free solution by injection into the anaerobic growth vessel 15 min prior to harvesting.

(c) Continuous Culture

The experiments involving growth of cells under chemostat conditions were carried out with the help of Dr Margaret Lowdon and Dr A. Lamb. All the chemostat cultures used glucose as the limiting growth factor. Since yeast type (respiratory-competent or respiratory-deficient) and the culture conditions (aerobic or anaerobic) affect the energy yield and hence the growth attained from the carbon source the glucose concentration in the feed media had to be varied appropriately. Thus, aerobic cultures of wild-type yeast were grown on 0.4 per cent glucose as carbon source in the feed media, while petite cultures or anaerobic cultures of the wild-type strain were grown with 2.0 per cent glucose. It was assumed that the energy yield under conditions where mitochondria were non-functional was one-fifth that where they were functional. The growth media for chemostat cultures was enriched to contain 1 per cent

(w/v) yeast extract and 0.1 per cent (w/v) peptone. Under these conditions, all cultures attained a steady state cell density of about 3 mg/ml.

The apparatus used for aerobic chemostat cultures is shown in figure 2-1 (a). Aeration was achieved by passing filtered air under pressure over the surface of the culture while it was rapidly stirred with a magnetic couple. The medium was inoculated and grown batchwise to late exponential phase (about 16 hrs), then the pump was started. Pumping rates for a given culture volume and for a desired generation time were determined from the following formula (Malek and Fenel, 1966).

$$D = \frac{F}{V} = \mu = \frac{0.693}{g}$$

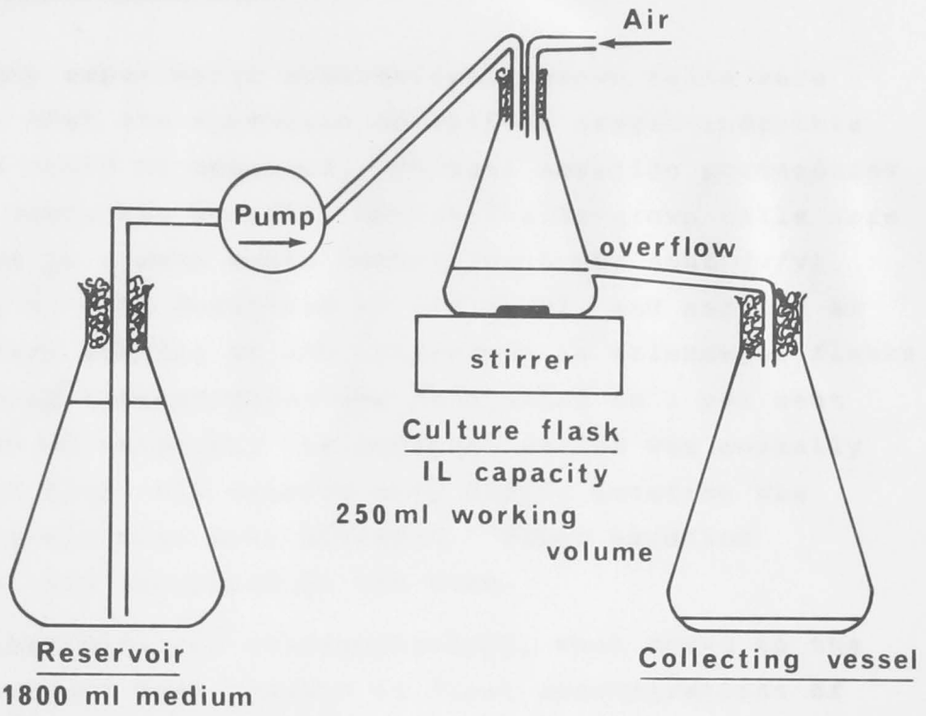
where D = Culture dilution rate (ml/hr)
F = Pumping rate (ml/hr)
V = Culture volume (ml)
μ = Exponential growth rate (hr^{-1})
g = Doubling time of culture (hr)

For a culture volume of 250 ml and a generation time of approximately 4 hrs, the pump rate used was 40 ml/hr. Samples of the outflow of the culture were monitored for glucose concentration, cell density, cytochrome content, and possible contamination. Under these conditions the steady state glucose concentration was <0.003 per cent.

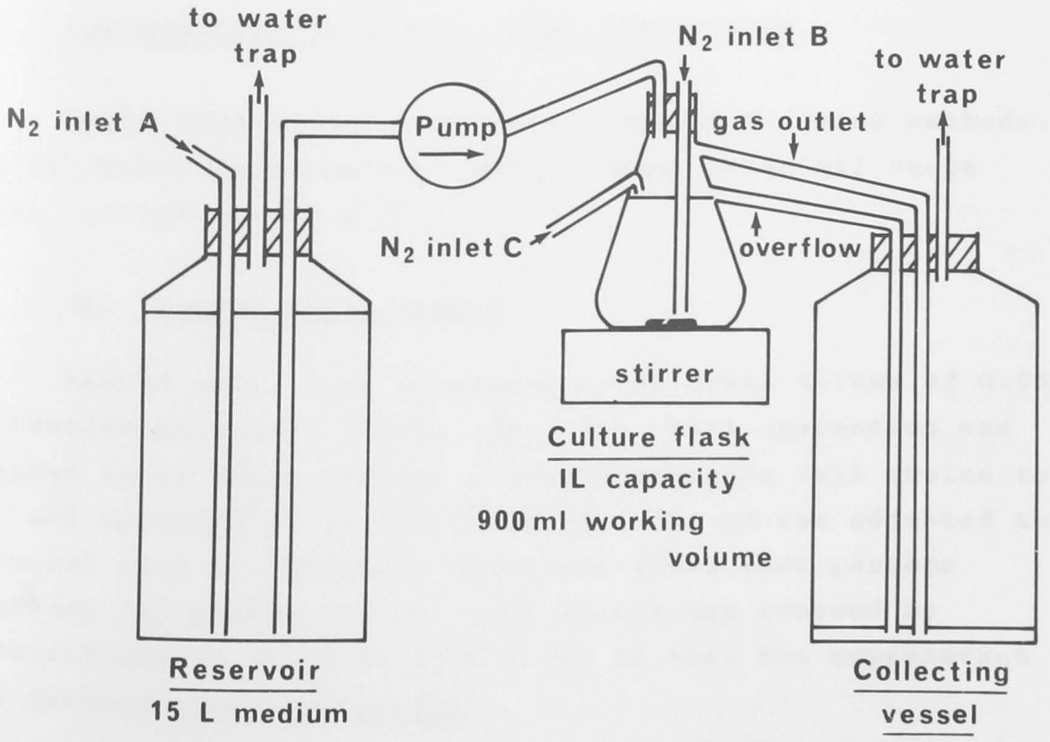
The apparatus was modified for anaerobic growth as shown in figure 2-1 (b). Both the reservoir and the culture flask were flushed with oxygen-free nitrogen, through inlets A and B, for 60 min prior to inoculation. After inoculation inlet B was closed : the nitrogen atmosphere of the culture was maintained though inlet C. Gas was allowed to escape via the water traps and all stoppers were sealed with paraffin wax. In other respects the procedure for anaerobic continuous culture was the same as that used for the aerobic cultures, except that the culture volume was increased to four-fifths that of the culture flask.

figure 2-1

a) Apparatus for Aerobic Continuous Culture



b) Apparatus for Continuous Culture under Anaerobiosis



3. AERATION CONDITIONS

In many experiments anaerobically-grown cells were aerated so that the synthesis of various oxygen-inducible components could be measured. Several aeration proceedures have been used, but normally anaerobically-grown cells were resuspended in growth media containing 1 per cent (w/v) galactose, at cell densities of 4-6 mg/ml, and aerated at 29° by rotary shaking at 300 cycles/min in erlenmeyer flasks. The galactose concentration was readjusted to 1 per cent after 4 hrs of aeration; the aeration period was normally 8 hrs. The high cell density used during aeration was designed to minimize cell division. Other aeration procedures are described in the text.

Cycloheximide and chloramphenicol, when added to the aeration medium, were present at final concentrations of 15-20 μM and 9 mM respectively.

4. PREPARATION OF WHOLE - CELL HOMOGENATES

Whole homogenates have been prepared by three methods, in different experiments. Except where noted all steps were carried out at 2-4°.

(a) French press method

Washed cells were suspended in an equal volume of 0.05 M potassium phosphate buffer (pH 7.4). This suspension was passed three times through a French pressure cell cooled to 2° and operated at 16,000 lb/sq in. The pH was adjusted to neutral with 1M potassium hydroxide after each passage through the French press. Cell debris was removed by centrifugation twice at 1000 g for 10 min; the supernatant is denoted whole homogenate.

(b) Spheroplast method

Spheroplasts were prepared by the snail enzyme method of Duell, Inoue, and Utter (1964) with modifications, including those of Kovac, Bednarova, and Grekshak (1968). Preincubation and snail enzyme digestion conditions are examined more completely in Appendix B. Packed spheroplasts were lysed by suspending in two volumes of cold 0.05M sorbitol - 1 mM phosphate / citrate (pH 7.0) and homogenisation in a Potter-Elvejhem homogeniser (5 strokes at low speed). The pH was adjusted to 7.0 with 1M potassium hydroxide, and the osmolarity was readjusted to 0.8 with 2M sucrose. Cell debris was removed by two centrifugations for 10 min at 1000g; the supernatant is denoted whole homogenate.

(c) Mechanical Method

This method is essentially that described by Schatz (1967). After harvesting, the cells were washed once with water and then with 0.5M sorbitol, 0.01M Tris-chloride, 0.5mM EDTA, pH 7.4 (STE buffer). The packed cells were resuspended in an equal volume of this buffer, an equal volume of glass beads (0.45 - 0.50 mm diameter) was added, and the slurry homogenised at high speed for 20 sec in a Braun MSK homogeniser with carbon dioxide as coolant. The broken cells were decanted from the beads, the beads were washed twice with an equal volume of STE buffer, and the washings added to the broken cell homogenate. Cell debris was removed as above. The supernatant is denoted whole homogenate.

5. PREPARATION OF MITOCHONDRIAL FRACTION

The mitochondrial fraction was spun from the whole homogenate, prepared by one of the above methods, by centrifugation at 10,000 - 15,000g (depending on the experiment) for 20 min. The mitochondria were washed with

STE buffer then resuspended in this buffer. In some experiments further purification of this fraction has been carried out, as described in the text.

6. PREPARATION OF RIBOSOMAL FRACTION

Cells were broken using the mechanical method, with the exception that breakage time was reduced to 15 sec. In some experiments cells were broken by grinding with alumina, but ribosome yield was low. A ribosomal pellet was prepared by centrifuging the post-mitochondrial supernatant at 192,000g av. for 60 min. The loosely-packed portion of the pellet was washed off and the hard-packed gelatinous portion retained and gently resuspended in 10 mM $MgCl_2$, 100 mM NH_4Cl, 10 mM Tris chloride, pH7.6 (AMT buffer) containing 10 mM β-mercaptoethanol. (For preparation of ribosomes the AMT/mercaptoethanol buffer was used throughout, rather than the STE buffer as described above).

7. PREPARATION OF pH 5 ENZYME FRACTION

The pH of the post-ribosomal supernatant was adjusted to five. After 30 min. at 0° the precipitate was sedimented (10,000 g, 5 min) and resuspended in AMT buffer. This suspension is denoted pH 5 enzyme fraction. It was found necessary to keep the whole homogenate as concentrated as possible, as dilution led to a marked decrease in the yield of pH 5 fraction.

8. MEASUREMENT OF RESPIRATION AND CYTOCHROMES

Respiratory activity of whole cells was measured polarographically at 30° in a Gilson oxygraph with a Rank electrode. The reaction mixture contained 100 μmoles potassium phosphate (pH 7.0), 20 μmoles glucose and 0.5-5 mg dry wt cells, in a final volume of 2.2 ml. Cyanide

sensitivity of the respiration of the cells was measured with potassium cyanide at a final concentration of 1mM.

Cytochrome spectra of whole cell suspensions were measured with a Cary 14R spectrophotometer using the method of Clark-Walker and Linnane (1967). Difference spectra of cell-free homogenates were obtained by the method of Estabrook and Holowinsky (1961), except that dithionite was used to reduce, and potassium ferricyanide to oxidize, the contents of the cuvettes. The amounts of cytochromes a, b and $c+c_1$ in these homogenates were obtained using the extinction coefficients of these authors. Cytochrome c was extracted from mitochondria by the method of Clark-Walker and Linnane (1967) and estimated by the method of Sels, Fukuhara, Pere, & Slonimski (1965).

9. ESTIMATION OF CELLULAR LEUCINE

The free leucine pool was extracted from cells using the fourth procedure of Thornton and McEvoy (1970). Leucine was estimated in this extract after separation of amino acids in a Beckman amino acid analyser.

10. EXTRACTION AND ESTIMATION OF UBIQUINONE

Attempts to extract ubiquinone from cells using chloroform-methanol or by Soxhlet extraction with acetone gave low irregular values, and instead a mild saponification method similar to that of Lester and Crane (1959) was used. Washed cells were suspended in water (at 50-100 mg/ml), an equal volume of 15 per cent (w/v) potassium hydroxide in ethanol and 0.5 g pyrogallol/gm dry wt cells were added, and the mixture boiled gently under reflux for 30 min. Saponification of sub-cellular fractions was carried out in a similar fashion. The protein solution (5 mg/ml) was added to an equal volume of the ethanolic potassium hydroxide with pyrogallol and heated at 80° for 10 min.

After cooling on ice, the non-saponifiable fraction was extracted into n-heptane (2 x 100 ml). The heptane extract was washed twice with an equal volume of water, and evaporated to dryness. The water washing of heptane extracts was essential for reproducible extractions; if this was omitted ubiquinone was apparently destroyed when the extract was concentrated . The residue was taken up in ethanol.

Purification of the ubiquinone from sterols and other components with absorption maxima in the UV was necessary for effective quantitative determination. For this purification, the crude extract was spotted onto a 20 x 20 cm plate of Kieselgel G (250 μ thick), previously activated by heating at 100° for 30 min. The developing solvent used was benzene : chloroform (1/1).

The ubiquinone extracted was identified by co-chromatography with authentic ubiquinone (Sigma) in the above solvent system, as well as in hexane : ethyl acetate (4/1). Ubiquinone purified in this way gave a characteristic difference spectrum and gave the same percentage reduction with potassium borohydride as did Sigma ubiquinone. TLC with a polar stationary phase, as described above, does not separate the isoprene homologues of ubiquinone. However, reverse-phase TLC does so (Wagner and Dengler, 1962), and showed that the ubiquinone obtained from this organism was ubiquinone - 30.

The quinone was determined quantitatively (after elution from thin-layer plates) by the absorbance change at 275 nm on reduction with potassium borohydride. The extinction coefficient used was $\Delta E^{1\%}_{1cm}$ (oxidized minus reduced) = 206 at 275 nm. Recovery from the chromatography step was approximately 90 per cent.

11. EXTRACTION AND ESTIMATION OF FATTY ACIDS AND ERGOSTEROL

A saponification step more vigorous than that used for extraction of ubiquinone was found to be necessary to obtain effective extraction of fatty acids and sterols. Saponification was carried out by adding 30 ml of 40 per cent (w/v) potassium hydroxide to a washed pellet containing approximately 1 g dry wt of cells (or by adding an equal volume of 20 per cent potassium hydroxide in the case of subcellular fractions) together with a known amount of pentadecanoic acid as internal standard for the fatty acid determination. Hydrolysis was carried out for 2 hr in a boiling water bath after which non-saponifiable lipids were extracted with diethyl ether (2 x 50 ml). The ergosterol content of this extract was estimated by the spectrophotometric method of Shaw and Jeffries (1953), directly in the case of anaerobic, lipid-supplemented cells or aerated cells. In the case of anaerobic, lipid-depleted cells, the non-saponifiable extract was in some cases first concentrated and chromatographed on silica gel plates in hexane-ethyl acetate (4:1, v/v), the ergosterol eluted with diethyl ether, and then estimated as described above. The chromatography step was found to be necessary because spectral traces of the non-saponifiable lipid fraction were frequently unsatisfactory as a basis for obtaining the extinction values to be used in the calculation. Recovery of ergosterol from the chromatography step was 70-75 per cent.

In some experiments the sterol content and distribution has been analysed by TLC and GLC. Two dimensional TLC was carried out on Kieselgel H plates, or Kieselgel G plates impregnated with 5 per cent silver nitrate (Morris, 1964). The solvent in the first direction was hexane : ethyl acetate (4/1) and in the second direction, chloroform. GLC analysis of the sterol-containing fraction was carried out on columns (5' x ¼") of 3 per cent OV17 or 3.3 per cent SE-30 on chromosorb CLP or diatoport S (Hewlett-Packard) at 250°. GLC peaks, or TLC spots, were identified in most cases by

comparison with known standards. In addition, the TLC spots were characterized by their staining properties with Lieberman-Buchard reagent (Cook and Rattray, 1958).

The fatty acids were extracted into ether (2 x 50 ml) after acidification of the residual hydrolysate to pH 1 with hydrochloric acid. After drying over anhydrous sodium sulphate the ether was removed under vacuum, and the fatty acids were methylated by refluxing with anhydrous methanol/HCl for 60 min at 50°. The fatty acids were then separated by gas-liquid chromatography on columns (6' x ¼") of 25 per cent polyethylene glycol adipate (Applied Science Laboratories) or 6 per cent diethylene glycol succinate (Hewlett Packard) on Chromasorb CLP or Diatoport S at 180°, and identified by their relative retention times (James, 1960). The columns were calibrated using standard fatty acid mixtures (Applied Science Laboratories).

12. EXTRACTION, SEPARATION, AND ESTIMATION OF PHOSPHOLIPIDS

(a) Extraction from whole cells

In preliminary experiments several extraction procedures were tested, including neutral and acid solvent extraction. Also, the efficacy of extraction after cell breakage was examined as several authors have reported that breakage prior to extraction is necessary for maximum lipid recoveries. These experiments showed that the method described by Letters (1968) gave the highest recoveries of phospholipid, as well as minimal lysophospholipid formation. In this method 50-200 mg of washed cells were resuspended with 1 ml of water and 4 ml of ethanol. The suspension was heated at 80° for 15 min, cooled, then centrifuged; the pellet was re-extracted twice with 5 ml of chloroform : methanol (2/1, v/v). Further extraction did not increase the yield of lipids. The extracts were combined, evaporated to dryness, and taken up in chloroform to a known volume (3-4 ml) : samples of this solution were used for phosphate

determination and for separation of phospholipid classes by TLC. Analysis of the fatty acid content of lipid-extracted cells and non-extracted cells indicated that lipid extraction was 70 per cent complete.

(b) Extraction from cell-free fractions

The fraction, usually mitochondrial, containing 10-20 mg protein at approximately 10 mg/ml was precipitated with 5 ml of 7.5 per cent TCA. After 1 hr at 0° the precipitate was centrifuged, washed twice with water, then extracted three times with 5 ml aliquots of chloroform : methanol (2/1) over a period of at least 2 hours, usually overnight. The extracts were combined and treated as in section (a) above.

(c) Thin layer chromatographic separation of phospholipid classes

Two-dimensional TLC was carried out essentially as described by Paltauf and Johnston (1970) on 20 x 20 cm plates (250 μ thick) of Kieselgel H (Merck). The exception was that the solvent for both directions was chloroform - methanol - ammonium hydroxide (65:35:5, v/v). In our experience the acid solvent described by Paltauf and Johnston (1970), as well as the acid solvent described by Getz, Jakovcic, Heywood, Frank and Rabinowitz (1970), caused the phospholipids to run very near the front. Phospholipids were identified by comparison with standards (Koch-Light) and by their staining behavior with ninhydrin (for phosphatidyl ethanolamine and phosphatidyl serine), Dragendorf reagent (for phosphatidyl choline, Wagner, Horhammer and Wolff, 1961), and molybdic acid spray (Wagner *et al.* 1961). Spots were scraped off the plates and the phospholipids digested without elution.

(d) Phospholipid digestion and estimation

Samples of the total lipid extract, or spots from the TLC plates, were transferred to centrifuge tubes and oxidized in the following way : 0.5 ml of oxidizing solution (2.5 M sulphuric acid, 1 M perchloric acid) and one drop of hydrogen peroxide were added to each sample and the samples heated in an oil bath at 170° for 30 min. After this time a further drop of peroxide was added to each tube and the heating was continued for at least 1 hr. After cooling the samples were made up to 1 ml with water, then 2 ml of ascorbic acid/molybdate reagent (1.7 per cent ascorbic acid, 0.4 per cent ammonium molybdate, 0.5M sulphuric acid) was added. The tubes were incubated at 45° for 20-45 min, together with a set of standards and controls which had been taken through the complete oxidizing procedure, and the colour read at 820 nm. The procedure of running standards through the oxidizing steps for each series of readings was necessary as the presence of oxidizing solution affected the colour development. Kieselgel, scaped off blank areas of the TLC plates, did not give any colour reaction. Also, colour was destroyed by peroxide, so it was important that all the peroxide had decomposed before phosphate assays were attempted.

13. *IN VIVO* INCORPORATION OF RADIOACTIVE LEUCINE AND URACIL INTO PROTEIN AND RNA, RESPECTIVELY

(a) *In vivo* incorporation during anaerobic or aerobic growth

At various times after inoculation samples were removed from the anaerobic growth vessel via a specially-fitted rubber septum to prevent ingress of oxygen. These samples were chilled in ice and their capacity to incorporate leucine and uracil was measured as follows. Approximately five mg of cells were quickly washed once with distilled water (20°) then resuspended in 0.05 M phosphate buffer containing 1 per cent galactose, also at 20°. The cell suspensions were

then incubated with shaking at 29° for 10 min, after which 0.3 ml of isotope solution (1 µCi ^{14}C - uracil, 10 µCi/µmole, and 0.2 µCi ^{3}H - leucine, 1 µCi/µmole) was added. Samples were removed at 0,5,10 and 20 min to 2 ml of cold 7.5 per cent TCA to stop the incorporation. Incorporation of leucine and uracil into aerobically-grown cells was carried out similarly. In some experiments, only ^{14}C-leucine was used as precursor. In more recent experiments, the washed cells were preincubated in the phosphate/galactose solution for five min under nitrogen. As well, the specific activity of the leucine was increased to 2 µCi/µmole and 0.6 µ Ci was added : the labelling time was decreased so that samples were removed at 0, 2, 4, and 8 min, and the labelling was carried out under nitrogen.

(b) *In vivo* incorporation during aeration

Samples of cells (approximately 5 mg) were removed from the aeration medium. In some experiments isotopes (as in section (a) above) were added directly to this cell suspension while in others the cells were first centrifuged, washed, and resuspended in galactose/phosphate (as above) before the isotopes were added. After the incorporation period the reaction was stopped as above. In some experiments only ^{14}C-leucine was used as precursor.

(c) Selective *in vivo* labelling and purification of (pro)mitochondria

This was carried out by a method similar to that described by Schatz and Saltzgaber (1969a). Cells were grown anaerobically, with or without lipid supplements, for 19-21 hrs. Cycloheximide to a final concentration of 10 µg/ml was then added in oxygen-free solution and incubation in the growth flask allowed to continue anaerobically for 15 min. Cells were then poured onto ice, harvested by centrifugation at 2°, washed with cold cycloheximide solution (10 µg/ml), and resuspended at 6-8 mg/ml in 0.05 M potassium phosphate (pH6.0) containing 1 per cent galactose and 25 µg/ml

cycloheximide. Erythromycin (5 mg/ml as Ilotycin, from Eli, Lilly and Co.) was added as indicated. Cell suspensions were shaken at 29° for 15 min under a stream of nitrogen then 50 µCi ^{3}H-leucine (34 Ci/m mole) was added and incubation was continued under nitrogen for 20 min. The incorporation of label was terminated by adding 2 ml of 70 mM DL-leucine (unlabelled) and immediately pouring the cells onto ice. The cells were harvested and promitochondrial fractions were prepared by the mechanical method detailed in section 4 (c). The promitochondrial fraction was washed three times with STE buffer, and resuspended in this buffer at approximately 10 mg protein/ml. The post-mitochondrial supernatant was centrifuged at 154,000 g for 60 min; the supernatant from this step was denoted the soluble fraction.

Promitochondria were loaded onto 30-70 per cent linear sucrose gradients containing 10 mM Tris-chloride and 0.5 mM EDTA (pH 7.0), and spun at 25,000 rpm for 60 min in a Spinco SW 25.1 head. Gradients were fractionated into 1 ml samples on an ISCO fractionator scanning at 254 nm. The extinction recorded was found to be predominantly due to light scattering. Bovine serum albumin (5mg) was added to each fraction, followed by 2 ml cold 7.5 per cent TCA containing 70 mM DL-leucine. After 1 hr at 0° the precipitate was collected, washed twice with the TCA - leucine solution, then resuspended in 1 ml of water and transferred to scintillation vials for counting. Protein from the soluble fraction was prepared by precipitation with TCA in the same way. Selective labelling of mitochondria (from aerobic cells) was carried out in a similar fashion.

14. PROCESSING OF WHOLE CELLS AFTER INCORPORATION

(a) Cells labelled with both uracil and leucine were collected, washed twice with 7.5 per cent TCA containing 70 mM DL-leucine, and resuspended in 1 ml of water.

(b) To remove incorporated uracil, or in the case of cells labelled with leucine only, samples were further processed by resuspension in 7.5 per cent TCA and heating at 85° for 15 min. After cooling, the cells were washed in TCA-leucine, then with acetone, and resuspended in ethanol : ether (2/1). The cells were then heated for a further 15 min at 65°, washed in ethanol : ether, then in ether, dried, and resuspended in water.

15. *IN VITRO* PROTEIN SYNTHESIS

Cytoplasmic ribosomes and supernatant enzymes were prepared from yeast, grown under various conditions, as described earlier. The incorporation procedure is based on that of Lamb, Clark-Walker, and Linnane (1968), and Bretthauer, Marcus, Chaloupka, Halvorson, and Bock (1963). The standard incubation mix contained in 1 ml : 35 mM Tris-HCl, pH 7.4, 140 mM NH_4Cl, 8.5 mM magnesium acetate, 2mM β-Mercaptoethanol, 1mM ATP (dipotassium salt), 5mM PEP, 30 µg (5E.U.) of pyruvate kinase, 0.2 µCi ^{14}C-leucine (40 µCi/µmole), and approximately 1 mg each of ribosomal protein and pH5 enzyme fraction. Cycloheximide and RNA were added as indicated. In earlier experiments GTP was added to the incubation but this was later found to be unnecessary. Incubations were usually for 0,5,10 and 20 min. The reactions were stopped by the addition of 5ml of 7.5 per cent TCA in 33 per cent ethanol (aqueous), followed by 2 mg of bovine serum albumin. After at least 1 hr at 0° the precipitates were collected, washed once with 7.5 per cent TCA containing 70 mM DL-leucine (unlabelled) then resuspended in this TCA-leucine solution and defatted as described above for cells. The defatting procedure was found to have little effect on the counts obtained (with the exception where lipids were added to the incubation), and so in most experiments this was omitted. In later experiments the precipitates were immobilized on glass fibre discs

(after the heating step(s)) where the washing steps were carried out. The precipitates were transferred to scintillation vials for counting as described below.

16. COUNTING PROCEEDURES

After processing a portion of the cells (resuspended in water), or the protein suspension, was transferred to a scintillation vial. Ten ml of Bray's scintillation solution was added and the cells or protein suspension counted in a Beckman LS-250 liquid scintillation counter using the automatic quench control. The composition of the scintillation solution was naphthalene (60 g), 2,5-diphenyloxazole(4g), 1,4 - di (2- (5- phenyloxazolyl)benzene (0.2 g), methanol (100 ml),ethylene glycol (20 ml), and 1,4 - dioxane to make one l. The remaining portion of cell suspension was diluted for optical density reading. Where both ^{3}H and ^{14}C incorporation was measured the ^{3}H counts have been corrected for overlap from the ^{14}C channel.

17. ELECTRON MICROSCOPY

This was carried out essentially as described by Wallace, Huang and Linnane (1968), with the following modifications. The fixation time in potassium permanganate was reduced to 30 min, and the cells were post-fixed with 10 volumes of 1 per cent osmium tetroxide, 0.05 M sodium phosphate (pH 6.8). In some cases (when protoplasts were fixed) the potassium permanganate step was replaced by a 2 hr (0^{o}) treatment with 3 per cent glutaraldehyde in 0.025 M sodium phosphate (pH 6.8).

After fixation the samples were twice washed with buffer then pelleted in 3/16" x 1 5/8" cellulose nitrate tubes. The portion of the tube containing the pellet was placed in a vial and dehydrated through an acetone series (the cellulose nitrate dissolves at high acetone concentration,

leaving the pellet). Alternatively the sample was sometimes pelleted then resuspended in a drop of just-liquid agar, and the agar block carried through the dehydration. Pellets were then embedded by firstly adding acetone : araldite (1/1, v/v) and over a period of 12 hours increasing the araldite concentration to 100 per cent. The pellet was finally set in a gelatine capsule in fresh araldite and hardened at 65° for at least 2 days. The pellet was then trimmed and thin sections cut using a glass knife with an LKB ultramicrotome.

Sections were placed on uncoated grids and stained with aqueous uranyl acetate and lead nitrate. The samples were then examined and photographed in an Hitachi HUll E electron microscope.

18. EXTRACTION OF RNA

The method used for the isolation of RNA from the subcellular fractions is a modification of the sodium deoxycholate-phenol method described by Click and Hackett (1966). Mitochondrial pellets, or cytoplasmic ribosomal pellets, were resuspended in 10 ml of buffered deoxycholate (0.1M sodium glycinate pH 8.0, 0.1M NaCl, 0.01M Na_2 - EDTA, 1 per cent sodium deoxycholate and 1 per cent bentonite) and homogenized for 2 min by shaking in a Braun shaker at low speed setting with carbon dioxide as coolant. 25 ml of phenol saturated with an aqueous solution of 1mM EDTA was added to the homogenate and the mixture shaken at 400 cycles/min for 10 minutes at 4°C. The mixture was centrifuged at 18,000 g for 5 min to resolve the aqueous and phenol phases. The aqueous phase was removed and extracted twice with ice-cold anhydrous diethyl ether. Two volumes of cold 95 per cent ethanol containing 10mM sodium acetate were added to the aqueous phase and cooled to -20° for 18 hr to precipitate RNA. RNA was recovered by centrifugation at 18,000 g for 30 min. The RNA pellet was dissolved in electrophoresis buffer (described below) containing 20 per cent (w/v) sucrose.

19. POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA

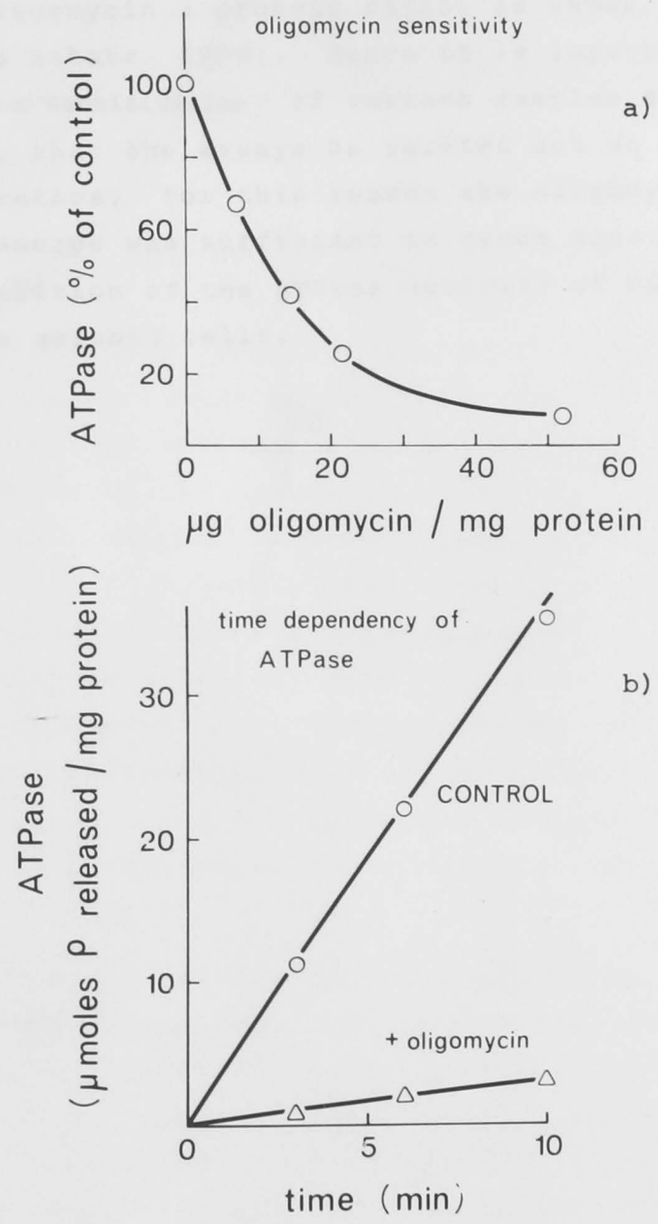
The RNA was subjected to polyacrylamide gel electrophoresis using the Tris-acetate-EDTA buffer system described by Loening (1969). The 2.4 per cent polyacrylamide gels of 9 cm length were prerun for 30 min before use. The RNA sample (10-30 µl, containing 20-60 µg RNA in electrophoresis buffer - 20 per cent (w/v) sucrose) was applied and electrophoresis was continued for 2 or 4 hours at 22°C and 5mA per gel. The gels were then removed from the plexiglass tubes and soaked in water for 1 hr. Absorption profiles were measured at 265nm using a Joyce-Loebl Chromoscan.

20. THE MEASUREMENT OF ATPase

ATPase (ATP phosphohydrolase, E.C.3.6.1.3.) was measured by following the rate of phosphate release from ATP. The incubation mixture contained in 2 ml : 6 mM ATP, 2 mM magnesium chloride, 16 mM Tris-chloride (pH 9.5), 80 mM sucrose and approximately 0.1 mg protein. Reaction (at 30°) was started by the addition of ATP after 5 min preincubation at 30°. After 10 min the reaction was stopped by addition of 0.4 ml 2M perchloric acid, and cooled to 0°. Samples were neutralised with 2M KOH, centrifuged, and aliquots of the supernatant assayed for phosphate as outlined in section 12 (d). The enzyme assay procedure is similar to that described by Kovac, Bednarova, and Greksak, (1968).

The mitochondrial ATPase from yeast is magnesium-dependent and has two pH maxima, the major one at pH 9.5 (Kovac *et al.* 1968, cf. mammalian mitochondrial ATPase which has a pH maximum at 7.5). There is disagreement as to whether the two pH maxima represent 2 enzymes (Kovac *et al.* 1968) or one enzyme (Somlo, 1968). Using the assay procedure described above, the reaction was linear with time (figure 2-2, a) and was proportional to protein

Figure 2-2



Mitochondrial ATPase from S. cerevisiae. Fig 2-2a shows the response of ATPase activity to oligomycin. Fig. 2-2b shows the time course of the ATPase reaction, with and without oligomycin (50 µg oligomycin/mg prot.).

concentration in the range used. It is worth commenting on the oligomycin-sensitivity, which is a characteristic of the membrane-bound enzyme. The inhibition by oligomycin depends on the oligomycin : protein ratio, as shown in figure 2-2 b, (see also Schatz, 1968). Hence it is important, if oligomycin sensitivities of various samples are to be compared, that the assays be carried out at similar inhibitor : protein ratios. For this reason the oligomycin concentration used in assays was sufficient to cause greater than 90 per cent inhibition of the ATPase activity of mitochondria from wild-type aerobic cells.

CHAPTER III

THE EFFECTS OF THE PETITE MUTATION AND CATABOLITE REPRESSION ON THE FORMATION OF YEAST MITOCHONDRIA: THE CONTROL OF UBIQUINONE SYNTHESIS IN YEAST

INTRODUCTION

1. THE REGULATION OF THE COMPONENTS OF YEAST MITOCHONDRIA

As previously detailed, the development of mitochondrial structure and activity in the yeast *Saccharomyces cerevisae* is controlled by environmental factors such as anaerobiosis and the quantity and nature of substrates available in the medium. For example, when wild-type cells of this organism are grown under anaerobic conditions functional mitochondria are not found and the activities of particulate mitochondrial enzymes such as succinate dehydrogenase and cytochrome oxidase are low or zero. Under aerobic conditions the development of functional mitochondria proceeds as reflected in the activity of these particulate enzymes : this development is repressed by fermentable substrates such as glucose.

Mitochondrial development is ultimately under the control of genes located in both mitochondria and nucleus. Particular interest has centred on the cytoplasmic petite mutation, a non-reversible, high-frequency mutation which is a result of a change in the mitochondrial genome and which leads to the inability of the cell to form functional mitochondria. The petite mutation is pleiotropic in that the synthesis of many mitochondrial components seems to be affected. The general conclusion drawn is that the biosynthesis of mitochondrial proteins in yeast is controlled by both environmental and genetic factors and that this regulation is common for many different mitochondrial components.

So far little attention has been directed at the control of the biosynthesis of non-protein components of mitochondrial

membranes. They include co-factors and prosthetic groups such as hemes, quinones and flavins, as well as lipids such as sterols and phospholipids. In this chapter some of the factors which regulate ubiquinone synthesis are examined. As described in the General Introduction, ubiquinone has been selected mainly because it appears to be a non-protein specifically located in mitochondria and is closely associated with the proteins of the electron-transfer chain.

A great deal of work has been carried out on the function of ubiquinone and its biosynthesis, but very little is known about how this synthesis is regulated. Obviously the regulation of cellular ubiquinone levels could be examined in terms of the activities and sites of the enzymes in the pathway. However these have not yet been characterized sufficiently. We are concerned mainly with the development of mitochondria, so the regulation of ubiquinone synthesis has been followed by studying the relationship between ubiquinone levels and the degree of mitochondrial development. The control of ubiquinone biosynthesis, from this point of view, can be examined relatively easily in *S. cerevisiae* since mitochondria in this organism can be subjected to a wide variety of environmental changes. As well, the availability of the cytoplasmic petite mutant enables genetic effects which result in modification of the organelle to be studied.

However, it is difficult to compare the biochemical properties of the petite organism and its parent wild-type, mainly because of differences in their response to the environment. Lack of consideration of these environmental effects seems to have led to many false conclusions concerning petite physiology, so it is worth examining these in more detail.

2. THE EFFECTS OF THE PETITE MUTATION ON MITOCHONDRIAL MORPHOLOGY: THE INVOLVEMENT OF CATABOLITE REPRESSION

In an early study of petite morphology (Yotsuyanagi, 1962) it was shown that cytoplasmic petites, in contrast to nuclear petites, had marked alterations in their mitochondrial structure; these yeasts were grown batchwise with 4 per cent glucose as carbon source. Avers and co-workers (Avers, Pfeffer, and Rancourt, 1965; Avers, Rancourt, and Lin, 1965) using a growth medium containing 1 per cent glucose, found effects similar to those reported by Yotsuyanagi (1962). Petites completely lacking cytochrome oxidase also lacked cristae in their mitochondria, while petites with low cytochrome oxidase contained mitochondria with cristae. However, because of the different growth conditions, these results are difficult to interpret.

Reilly and Sherman (1965) and Mounolou, Jakob and Slonimski (1966) were the first to emphasise that in order to demonstrate correctly differences in mitochondrial constitution between petite and wild-type cells,it is necessary to grow the cells under conditions that obviate environmental effects. This necessity arises because of effects on development due to catabolite repression. Petite cells can grow only in the presence of a fermentable substrate, i.e. under conditions which cause catabolite repression. If petite and wild-type cells respond differently to catabolite repression (as appears to be the case, see below), then a valid comparison can only be made when effects due to catabolite repression are minimal. The problem becomes one of differentiating between effects that are due directly to the genetic lesion and effects that are due to a selective catabolite repression.

Bowers, McClary and Ogur (1967) demonstrated the importance of glucose repression in their studies on mitochondrial morphology. They found that a petite culture grown batchwise with 2 per cent glucose as carbon source had non-cristate mitochondrial profiles, while the same

mutant grown in a chemostat on limiting glucose (so that repression was minimized) showed normal mitochondria. Smith, Marchant, Maroudas and Wilkie (1969) have also considered the importance of catabolite repression. This group grew various petite strains batchwise on melibiose, a substrate which causes little repression (Reilly and Sherman, 1965), and found effects similar to those of Bowers *et al.*, (1967) although the petites examined showed a 30 per cent decrease in the number of cristate mitochondria.

These results suggest that cytoplasmic petite cells are very sensitive to catabolite repression. Nevertheless, the sensitivity of the petite mutants has not been explained in any detail. In a recent review of the phenomenon of catabolite repression (Paigen and Williams, 1970) the authors attribute the action of many metabolic inhibitors largely to catabolite repression. Their general conclusion is that in organisms sensitive to catabolite repression any agent which restricts the utilization of catabolites, but does not restrict their influx, will cause a catabolite repression of sensitive pathways. For example, glucose, which is rapidly fermented, would be expected to cause a particularly intense repression if utilization of its catabolites were restricted. These considerations have obvious relevance to petite mutants, since the oxidation of glcolytic end-product is severely restricted in them. Because wild-type *S. cerevisiae* is classically susceptible to catabolite repression (see General Introduction), then even severer catabolite repression is to be expected in the petite mutant of this organism. In fact the observations on the petite mitochondrial structure discussed earlier can be seen in this context.

3. THE EFFECT OF THE PETITE MUTATION ON UBIQUINONE SYNTHESIS

The difficulty of comparing wild-type and petite cells is further illustrated when ubiquinone synthesis is examined. Sugimura, Okabe, and Rudney (1964) found low levels of ubiquinone in petite yeast, and suggested that this might be due to a genetic defect in the synthesis of an oxygenase in the ubiquinone pathway. Mackler, Douglas, Will, Hawthorne, and Mahler (1965) found that electron-transfer particles derived from petites contained lower ubiquinone levels (they claimed to rule out possible differences due to glucose repression by growth on several different carbohydrates, including sucrose and galactose). They proposed that the low levels of ubiquinone in these particles was due to incomplete assembly. However, whole cell levels were not examined to test this possibility.

In the following sections the effect of the petite mutation on ubiquinone synthesis has been reexamined as the discussion presented earlier suggested that the growth conditions are very important.

It is clear that any comparison of the petite and wild-type strains of yeast must also consider the effect of catabolite repression, and possible differences in the degree of repression. Perhaps the most valid comparison of these two strains can be made after growth in a chemostat under conditions of carbon source limitation. In a chemostat new medium is continually added to the culture vessel and an equivalent volume removed: the organisms in the culture adjust their growth rate to the concentration of some limiting nutrient, so that the culture density becomes constantwhen the steady state is reached. Within limits, the cell generation time is determined by the rate of addition and removal of new medium, i.e. by the dilution rate, while the cell density is determined by the concentration of limiting nutrient in the added medium. Thus a chemostat can be used to maintain a constant culture environment.

Also, if the limiting nutrient is the carbon source then any catabolite repression due to this carbon source is minimized, as its concentration at the steady state is vanishingly small. The principles of continuous culture are discussed, for example, by Herbert, Elsworth and Telling (1956), Herbert (1961), and Powell (1965). Chemostat cultures have been used in some experiments to restrict effects due to catabolite repression.

The following sections present experiments designed to define some of the effects of growth conditions and strain of the organism on the cellular level of ubiquinone. Some experiments on the incorporation of labelled precursors into ubiquinone are also presented. In later chapters the response of other lipid pathways will be considered.

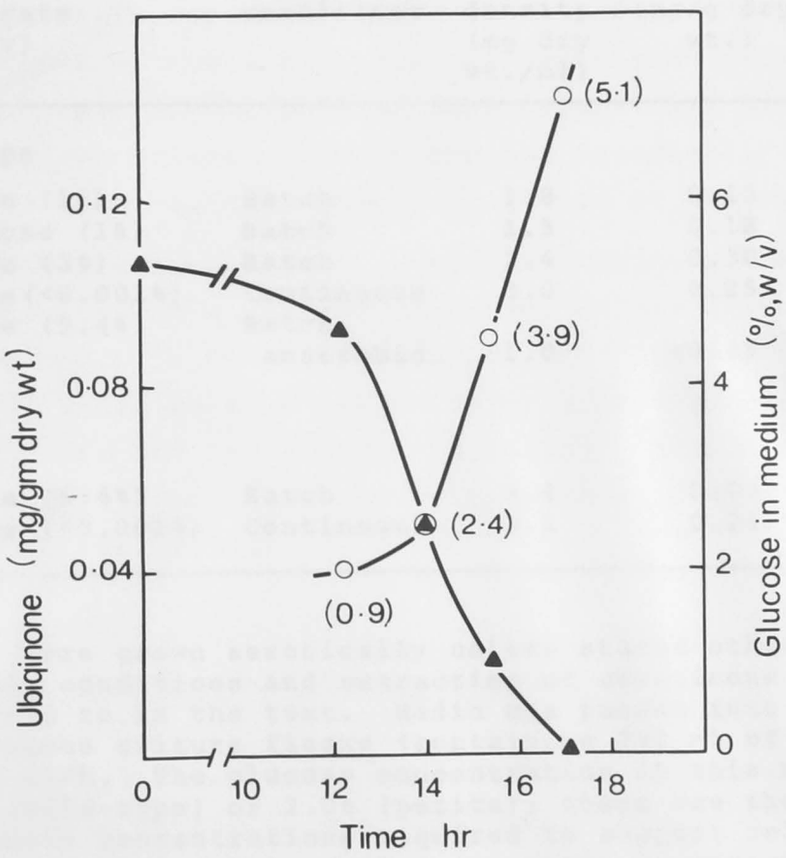
RESULTS AND DISCUSSION

1. UBIQUINONE FORMATION IN WILD-TYPE YEAST : THE EFFECT OF CATABOLITE REPRESSION

An initial observation was that wild-type cells grown through many divisions (in batch culture with glucose as carbon source) contained more ubiquinone than younger cultures. It was found that this could be related to the glucose concentration. As illustrated in figure 3-1, there was an inverse relationship between glucose concentration in the medium at the time of harvest and ubiquinone concentration when the wild-type strain was grown batchwise with 5.4 per cent glucose as the initial concentration of carbon source. The highest concentrations of ubiquinone under these growth conditions were observed after the glucose was exhausted.

The effects of growth conditions and the nature of the carbon source are examined in the experiments reported in Table 3-1 where the level of ubiquinone and cytochrome oxidase are compared. As earlier workers have shown, (Lester and Crane, 1959; Sugimura and Rudney, 1960) anaerobic growth results in low ubiquinone levels. In fact,under our anaerobic growth conditions ubiquinone is not detectable. Aerobic growth results in the synthesis of ubiquinone,

Figure 3-1



The relationship between glucose concentration in growth media and cellular ubiquinone content. Cells were grown on 5.4% glucose media from the same initial inoculum (0.005 mg dry wt./ml medium) for different periods to give different cell yields. ▲—▲ , glucose concentration in the medium at the time of harvest; O—O , ubiquinone content of the cells. Figures in parentheses are the cell densities (mg dry wt. cells/ml medium) at the time of harvesting.

TABLE 3-1

EFFECT OF GROWTH CONDITIONS ON UBIQUINONE CONTENT AND CYTOCHROME OXIDASE ACTIVITY OF *S. CEREVISIAE*

Growth Substrate (w/v)	Culture conditions	Cell density (mg dry wt./ml)	Ubiquinone (mg/g dry wt.)	cytochrome oxidase
Wild-type				
Glucose (1%)	Batch	1.8	0.13	190
Galactose (1%)	Batch	1.5	0.18	-
Lactate (3%)	Batch	2.4	0.30	475
Glucose (<0.001%)	Continuous	3.0	0.25	350
Glucose (5.4%)	Batch, anaerobic	1.0	<0.01	3
Petite				
Glucose (5.4%)	Batch	2.4	0.02	<1
Glucose (<0.001%)	Continuous	3.2	0.24	<1

Cells were grown aerobically unless stated otherwise. Culture conditions and extraction of ubiquinone are referred to in the text. Media was pumped into the continuous culture flasks (containing 250 ml of medium) at 45 ml/h. The glucose concentration in this media was 0.4% (wild-type) or 2.0% (petite); these are the substrate concentrations required to support cell densities of 3.0-3.2 mg/ml under these conditions. The steady-state glucose concentration in the continuous culture was not detectable using the Glucostat reagent (Worthington Biochemicals). Cytochrome oxidase activity is expressed as mμmoles cyt. c oxidised/min/mg protein.

but as Table 3-1 shows, this is modulated by the type and amount of carbon source present. The concentration of ubiquinone in cells grown in batch culture, and harvested within one cell-division of exhaustion of the sugar, was always significantly greater when galactose was used instead of glucose. However, the highest ubiquinone levels were observed in cells grown on the non-fermentable substrate lactate, where catabolite repression is minimized (Polakis and Bartley, 1965). When wild-type cells were grown in a chemostat so that glucose was growth-limiting and catabolite repression very low, it was found that the ubiquinone level approached that of the lactate-grown cells. Glucose was not measurable (less than 0.001 per cent) in the chemostat culture at harvest.

The variations in the level of ubiquinone closely mimic the variations of a protein component of the electron-transfer chain, i.e. cytochrome oxidase. Thus there are very low levels of this enzyme after anaerobic growth whereas after aerobic growth the highest levels are observed under conditions where catabolite repression is negligible (Table 3-1).

2. UBIQUINONE FORMATION IN PETITE YEAST

Table 3-1 also shows the effects of growth conditions on ubiquinone levels in a petite mutant of the wild-type strain. As expected, the mutant contained negligible cytochrome oxidase under repressed or derepressed conditions. When this petite was grown aerobically in batch culture with 5.4 per cent glucose as the carbon source the cells were found to contain low concentrations of ubiquinone after growth to stationary phase, although this level was significantly greater than that found in anaerobically-grown cells. Similar results have been previously reported, as outlined in the introduction to this chapter, (Sugimura *et al.* 1964; Mackler *et al.* 1965).

However, in view of the likely complication of catabolite repression, we have also measured the ubiquinone content after the petite had been grown in a chemostat under conditions of glucose limitation. It can be seen that cells, wild-type or petite, grown under these conditions contain the same high level of ubiquinone. (Reverse-phase TLC showed that the petite ubiquinone was the same homologue, ubiquinone-30, as that found in the wild-type). Thus the low level of ubiquinone found in petite cells grown in batch culture is a consequence of a very high degree of catabolite repression, and is not a direct effect of a genetic change in the ubiquinone pathway.

This is in contrast to the effect of the petite mutation on cytochrome oxidase activity, as this enzyme is not formed in the petite even under conditions of derepression. It is apparent (see General Introduction) that the cytoplasmic petite mutation results in the selective deletion of the synthesis of particulate cytochromes and respiratory enzymes. The results presented above for cytochrome oxidase synthesis, and in a later chapter for other mitochondrial enzymes, show that this is not due just to increased catabolite repression. On the other hand the biosynthesis of mitochondrial components such as cytochrome c (Slonimski, 1956), mitochondrial DNA (Mounolou *et al.* 1966; Moustacchi and Williamson, 1966; c/f Fukuhara, 1969) and ubiquinone is affected at the level of control, for example by catabolite repression. Experiments on the oxygen-induced syntheses of mitochondrial components, to be discussed in a later section, show that it is possible to obtain a similar dissociation of ubiquinone synthesis and respiratory enzyme syntheses by the use of specific inhibitors of protein synthesis.

It is interesting to note that the normal formation of ubiquinone in the petite (under conditions of derepression) implies that the enzymes of this pathway are formed by a non-mitochondrial protein-synthesising system. This follows from recent findings that cytoplasmic petites have no functional mitochondrial protein-synthesising system (see Chapter I).

The finding that catabolite repression, as well as oxygen tension, markedly affects the level of ubiquinone in wild-type yeast cells indicates that the formation of this non-protein component of the electron-transfer chain is normally controlled in a manner similar to that of enzymic components of the chain. This can be seen by the comparison with cytochrome oxidase levels. If the cytochrome oxidase activity is taken to reflect the extent of mitochondrial development, then it is clear that ubiquinone levels also reflect this development.

3. STUDIES ON THE INCORPORATION OF LABELLED PRECURSORS INTO UBIQUINONE

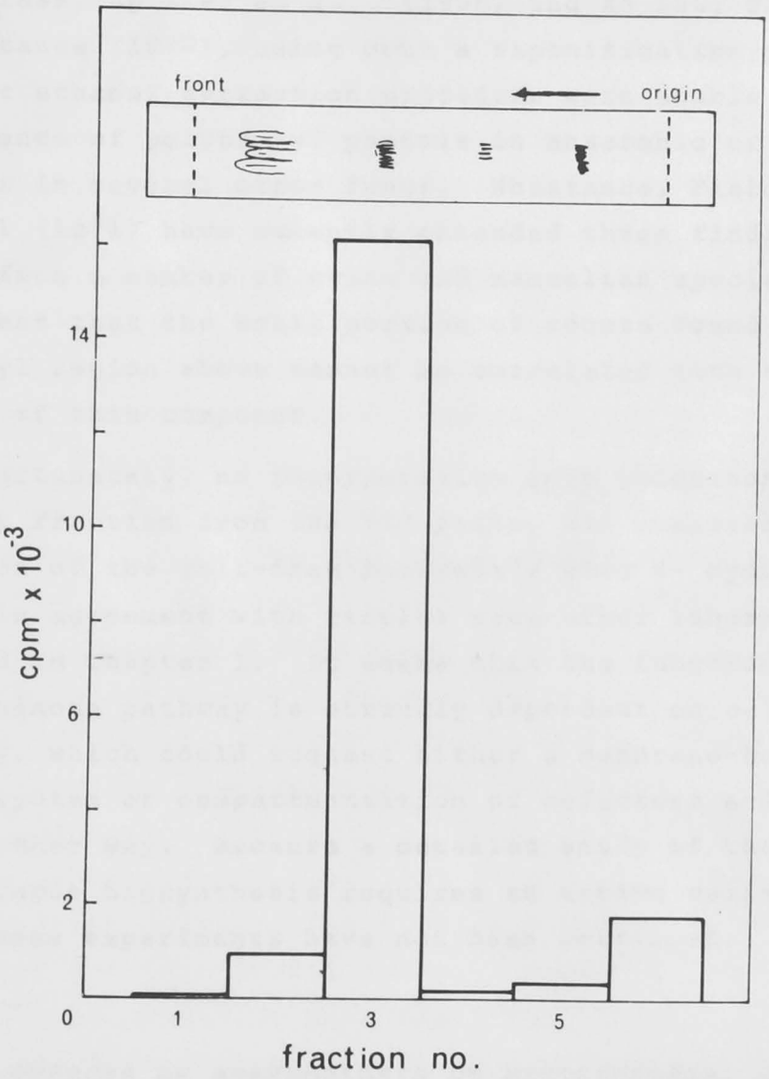
It was felt that questions concerning the more detailed control of ubiquinone synthesis in yeast could not be examined without a more sensitive indicator of synthesis than net quinone formation, as described above. Preliminary experiments on the incorporation of ^{14}C-benzoate into ubiquinone were therefore undertaken in the hope that a radioassay procedure might provide such a tool. However, benzoate proved to be an unsatisfactory precursor in the organism. For example, when anaerobically-grown yeast were aerated in the presence of this label only 0.13 per cent of the counts were incorporated into ubiquinone, and a comparison of the specific activities of the added label and the purified ubiquinone indicated that extensive isotope dilution (approximately 1:10^4) had occurred. This may have been due to high endogenous levels of ubiquinone precursors or intermediates in the anaerobic cell, impermeability of the cell to the label, or to a combination of these effects. Subsequently we have tested ^{14}C-4- hydroxybenzoate, as this compound has been found to be efficiently converted to ubiquinone in yeast (Rudney and Parson, 1963; Parson and Rudney, 1965; Spiller, Threlfall, and Whistance, 1968).

In a typical experiment, wild-type cells were grown with glucose as carbon source to mid-log phase (aerobically or anaerobically), harvested and washed, then resuspended in 0.25M potassium phosphate (pH 7.0) containing 1.2 per cent glucose. A portion of the cells was broken in the Braun shaker and a whole homogenate prepared as described in Chapter II. Both the cell-free homogenate and the cells were then aerated (separately) for 4 hours in the presence of ^{14}C(U)-4- hydroxybenzoate after which ubiquinone and other non-saponfiable lipids were extracted. This crude extract was fractionated on TLC plates with or without carrier ubiquinone, and the various bands eluted for counting and ubiquinone estimation.

The separation pattern, as it appeared under UV light, of the extract from whole cells after incorporation is shown in figure 3-2. Fractions 1 and 6 were the front and origin of the plate, respectively; fraction 3 was ubiquinone and fraction 5, ergosterol. Fraction 2 contained a UV-fluorescent compound(s). Nearly all (90 per cent) of the counts were recovered in the ubiquinone band. The absence of radioactivity from ergosterol, the skeleton and methyl substituents of which are derived from the same precursors as the prenyl side chain and methyl groups of ubiquinone, indicate that 4- hydroxybenzoate is only incorporated into the ubiquinone nucleus, and that this precursor is most probably incorporated *in toto*. A small portion of the recovered counts were found in band 2, the region where polyprenyl phenol would be expected to run.

Separate experiments were designed to isolate any polyprenyl phenol present. Cells were grown anaerobically as this phenol, a ubiquinone precursor, has been found to accumulate under similar circumstances in other organisms, for example in *Rhodospirillum rubrum* (Rudney and Raman, 1966) and *E. coli* (Whistance, Brown and Threlfall 1970). The normal saponification procedures were applied, and the TLC chromatogram examined for polyprenyl phenol using both a diazo spray reagent and spectra. No polyprenyl phenol

Figure 3-2



Incorporation of ^{14}C-4-hydroxybenzoic acid into ubiquinone. Cells were labelled as described in the text, and a crude ubiquinone (non-saponifiable) fraction extracted. The crude extract was concentrated, then separated by TLC as shown above. Bands shown were viewed under UV light; ubiquinone and ergosterol bands were further identified by co-chromatography with standards, and by UV spectra.

could be detected. However in recovery experiments it was found that most (approximately 80 per cent) of any added octaprenyl phenol was destroyed by the saponification procedure, so the sensitivity of the method is probably low. Nevertheless, Spiller *et al.* (1968) and Ah Law, Threlfall and Whistance (1970), using both a saponification procedure and a hot ethanol extraction procedure were unable to detect the presence of polyprenyl phenols in anaerobic or aerobic yeast, or in several other fungi. Whistance, Field and Threlfall (1971) have recently extended these findings to tissues from a number of avian and mammalian species. It is apparent that the small portion of counts found in the polyprenyl region above cannot be correlated with the presence of this compound.

Unfortunately, no incorporation into ubiquinone, or any other fraction from the TLC plate, was observed after incubation of the cell-free homogenate with 4- hydroxybenzoate. This is in agreement with results from other laboratories, discussed in Chapter I. It seems that the functioning of the ubiquinone pathway is strongly dependent on cellular integrity, which could suggest either a membrane-bound enzymic system or compartmentation of cofactors and enzymes in some other way. Because a detailed study of the control of ubiquinone biosynthesis requires an active cell-free system these experiments have not been continued.

4. THE EFFECTS OF ANAEROBIOSIS ON MITOCHONDRIAL ENZYMES AND CYTOCHROMES : DISTINCTION BETWEEN CATABOLITE REPRESSION AND ANAEROBIOSIS.

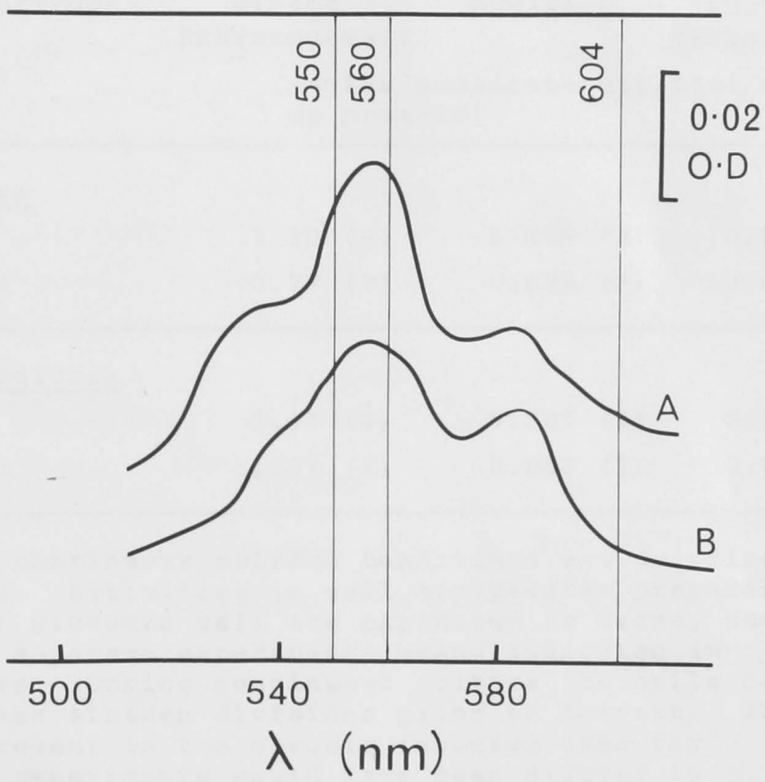
It is established that the synthesis of mitochondrial enzymes and cytochromes does not occur in the absence of oxygen. However, it has been suggested that this lack of synthesis is due to catabolite repression rather than the lack of oxygen (Polakis, Bartley, and Meek, 1964, 1965; Tustanoff and Bartley, 1964; Singer, Rocca, and Kearney, 1966;

Chapman and Bartley, 1968). As discussed in a previous section, petite cells are subject to a high degree of repression in batch culture. This is because any set of environmental conditions which (by restricting the flow of metabolites) augments the concentration of repressor molecules, will cause increased repression. Clearly, wild-type yeast cells grown under anaerobic conditions are affected by a restriction on carbon source utilization similar to that in petite cells, so the postulate cited above is quite plausible.

We sought to distinguish between the effects of anaerobiosis and the effects of catabolite repression by growing cells anaerobically in a chemostat under conditions of glucose limitation so that catabolite repression was minimized. The apparatus and media used for this purpose has been described in the methods section. Figure 3-3 shows the whole-cell cytochrome spectra from anaerobic, lipid-supplemented cells grown batchwise and in continuous culture. No aerobic cytochromes were found in either culture. The enzyme levels of cells grown anaerobically, batchwise and in the chemostat, are shown in Table 3-2; those of cells grown aerobically are included for comparison. Aerobic growth in continuous culture led to considerable derepression of malate dehydrogenase, relative to the level after aerobic growth in batch culture; the other enzymes examined were also derepressed but to a lesser extent. Anaerobic growth under either culture condition gave the same low value for fumarase and succinate dehydrogenase activities, although there appeared to be some derepression of malate dehydrogenase. Nevertheless there was still a marked inhibition of the formation of malate dehydrogenase by anaerobic conditions.

The results show that the absence of aerobic cytochromes and the decreased levels of respiratory enzymes observed when cells are grown anaerobically is a consequence of the lack of oxygen, and that these effects are not due to catabolite repression, even though it is likely that this is increased under anaerobic conditions. This finding agrees with that of Somlo and Fukuhara (1965), who used a different experimental approach.

Figure 3-3



Whole cell cytochrome spectra of cells grown anaerobically, under continuous culture or batch culture conditions. Curve A : continuous culture cell spectra. Curve B : batch culture cell spectra. The positions of the absorption bands of cytochromes of aerobic cells are indicated.

TABLE 3-2

THE EFFECT OF ANAEROBIOSIS ON THE ACTIVITY OF THE TCA-CYCLE ENZYMES IN CELLS DEREPRESSED IN CONTINUOUS CULTURE

CULTURE CONDITIONS	MALATE DEHYDROGENASE	FUMARASE	SUCCINATE DEHYDROGENASE
	(μmoles substrate utilized/minute/mg protein)		
Batch culture			
Aerobic	1.20 (6)	0.138 (3)	0.126 (3)
Anaerobic	0.77 (3)	0.080 (3)	0.025 (3)
Continuous culture			
Aerobic	2.67 (4)	0.207 (4)	0.186 (4)
Anaerobic	1.39 (2)	0.080 (2)	0.029 (2)

Batch and continuous culture conditions are described in Chapter II. Activities in cell homogenates prepared with the French pressure cell are expressed as means, the number of separate experiments being indicated in parentheses. During continuous culture the cells carried out at least sixteen divisions prior to harvest. Thus enzymes present in the aerobic inoculum used for anaerobic experiments would have been diluted to insignificant activities at the time of harvesting the anaerobic cells. Values presented are the results of assays carried out by Dr. M. Lowdon.

CHAPTER IV

THE EFFECTS OF LIPID DEPLETION ON ANAEROBICALLY-GROWN YEAST

INTRODUCTION

1. AN EXAMINATION OF THE PROPERTIES OF PROMITOCHONDRIA

In spite of early controversy (see Chapter I) it now appears reasonably certain that cells of *Saccharomyces cerevisiae* when grown under strictly anaerobic conditions contain mitochondria-like structures, and that this is true whether the anaerobic growth medium contains lipid growth factors in limiting or non-limiting amounts. Even so, these promitochondria are largely non-functional, as they lack the cytochromes and respiratory enzymes of mitochondria from aerobic yeast and so have negligible respiration. The question of the biological role of promitochondria is an interesting one. It is possible that these organelles are completely inactive in the economy of the anaerobic cell : the preservation of their structure may only be a mechanism to allow the rapid formation of functional mitochondria on the introduction of oxygen. Alternatively it is possible that promitochondria retain biological activity that is not related to respiration or oxidative phosphorylation, for instance as a locus of metabolic transformations involving TCA-cycle enzymes.

Most work on the biochemical characterisation of promitochondria has been carried out with organelles isolated from lipid-supplemented cells. It has been found that these contain DNA (Fukuhara, 1967b; Criddle and Schatz, 1969) and an active protein-synthesising system (Davey, Yu and Linnane, 1969; Schatz and Saltzgaber, 1969a; Watson, Haslam, Veitch, and Linnane, 1971) suggesting that these organelles are biosynthetically active in the anaerobic cell. The fact that the organelles contain DNA suggests that they may be sites for the conservation of part at least of the genetic information that governs mitochondrial development in

aerobically-grown cells (Wilkie, 1968; Criddle and Schatz, 1969; Rabinowitz, Getz, Casey, and Swift, 1969; Thomas and Wilkie, 1968). Promitochondria from lipid-supplemented cells also contain oligomycin-sensitive ATPase (Schatz, 1963, 1965; Criddle and Schatz, 1969) as well as a Pi - ATP exchange reaction that is sensitive to uncouplers, rutamycin, and atractyloside, and a H_2O - Pi exchange reaction sensitive to rutamycin and the uncoupler 1799 (Groot, Kovac and Schatz, 1971), though these activities are much less than those found in mitochondria from aerobic cells. Promitochondria apparently contain an energy-transfer system that is similar to that of normal mitochondria, in spite of their lack of aerobic cytochromes ($\underline{aa}_3$, $\underline{b}$, $\underline{c}$, and $\underline{c}_1$,) and ubiquinone. It has been pointed out by Groot *et al.* (1971) that this represents the first instance of a mitochondrial energy-transfer system that is dissociated from a respiratory chain.

Groot *et al.* (1971) also describe experiments that relate the formation of the energy-transfer system and the biosynthetic capacity of promitochondria. When wild-type cells were grown in the presence of erythromycin, an inhibitor of mitochondrial protein synthesis, the isolated promitochondria could not catalyse the Pi - ATP exchange reaction. The petite mutation, which has as one consequence the loss of mitochondrial (or promitochondrial) protein-synthesising activity(Kuzela and Grecna, 1969; Schatz and Saltzgaber, 1969a) also results in the loss of the Pi - ATP exchange reaction. The conclusion is that a component(s) of the energy-transfer system is formed by the mitochondrial protein-synthesising system, i.e. not only do promitochondria from lipid-supplemented cells contain a functional mitochondrial protein-synthesising system, but the system is also active in the anaerobic cell in the formation of the energy-transfer or coupling complex.

2. THE EFFECTS OF LIPID-DEPLETION DURING ANAEROBIC GROWTH

The effect of lipid depletion on the metabolic activity of promitochondria remains uncertain. *S. cerevisiae* is normally considered to be a facultative anaerobe, even though prolonged anaerobic growth depends on an exogenous supply of usaturated fatty acids and sterol, the biosynthesis of which requires molecular oxygen. It might be expected that the omission of essential growth factors such as these lipids from the anaerobic growth medium would result in considerable changes in the physiology of the cells.

Several effects have already been seen at the whole cell level. Differences between anaerobic cells grown with and without lipid supplements include lipid composition (Klein, 1955; Kovac, Subik, Russ and Kollar, 1967; Jollow, Kellerman, and Linnane, 1968) and the nature of the cytochromes or other pigments present in the cell (Wallace, Huang and Linnane, 1968). The adaptive response to oxygen of each of these two cell types is also characteristic, particularly with regard to the sensitivity of the induced enzyme and lipid syntheses to inhibitors of protein synthesis (Vary *et al.* 1970). These effects are described in detail in Chapter V.

More recently some of the differences between lipid-supplemented and lipid-depleted anaerobes have been examined at the promitochondrial level. Watson *et al.* (1971) have reported that promitochondria isolated from lipid-depleted cells do not contain an active mitochondrial protein-synthesising system, in contrast to those from lipid-supplemented cells. This is a key observation, and is consistent with the whole cell experiments of Vary *et al.* (1970), which led these workers to postulate such an effect of lipid depletion. Consideration of the findings of Groot *et al.* (1971) discussed above, would lead to the prediction that promitochondria from lipid-depleted anaerobic cells, if they lack mitochondrial protein synthesis, would be unable to form a mitochondrial energy-transfer system; this is discussed in the next chapter. Forrester, Watson and Linnane

(1971a) have recently claimed that lipid-depleted promitochondria lack mitochondrial ribosomal RNA.

However, promitochondria from lipid-depleted anaerobes have an unusual lipid composition (Paltauf and Schatz, 1969; Chapter V) suggesting altered membrane properties. It is possible, for example, that the isolation of promitochondria from lipid-depleted cells results in fragmentation of the organelle. Special precautions, involving pretreatment with glutaraldehyde, have been taken by some workers (Damsky, Nelson and Claude, 1969; Watson *et al.* 1971) during isolation of promitochondria from these cells. However, it is possible that this treatment may inactivate many enzymes, including those essential for protein synthesis, so that the difficulties of isolation of lipid-deficient promitochondria could be the cause of their inactivity in this respect.

The following section describes experiments relating to the effects of lipid depletion/supplementation on the anaerobic growth physiology of the cells and their behaviour during aeration. In view of the work discussed above the study has included an examination of the protein and RNA synthetic capacities of the cells during anaerobic growth. In order to define the changes which occur more precisely, these have been extended to a study of *in vitro* protein synthesis in cytoplasmic ribosomal systems derived from both types of anaerobic cell, as well as an examination of the ribosomes and of ribosomal RNA. Results on the ability of anaerobically-grown cells to incorporate labelled precursors into RNA and protein during adaption to aerobic conditions are also presented. Questions concerning the relationship of promitochondria to the mitochondria found in aerobic cells are considered in the following chapter.

RESULTS

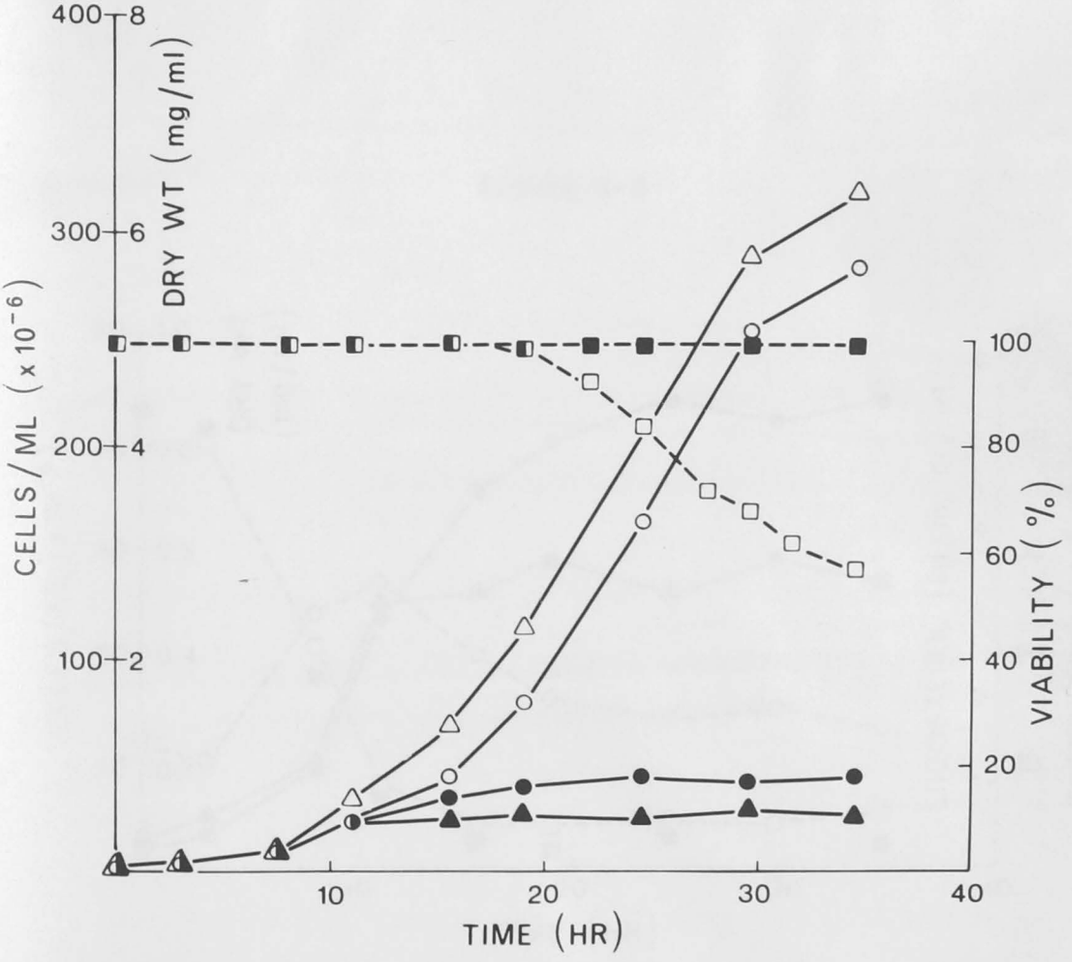
1. THE EFFECTS OF THE MEDIUM COMPOSITION ON THE GROWTH PHYSIOLOGY AND LIPID CONTENT OF CELLS DURING ANAEROBIC GROWTH

The growth of *S. cerevisae* under anaerobic conditions, with and without lipid supplements in the medium, is shown in figure 4-1. In cultures grown without lipid supplements cell division ceased about 11 hr after inoculation even though in the subsequent 12-14 hr cell mass, measured turbidimetrically or gravimetrically, approximately doubled. By comparison, the cells of lipid-supplemented cultures divided and grew continuously over the entire period: growth ultimately ceased due to exhaustion of the carbon source.

The viability of the two types of culture, as shown by the capacity of the cells to exclude methylene blue (Gurr, 1965) was dependent on the supply of lipids. Shortly after cell mass stopped increasing in the lipid-depleted cultures cell viability began to decline, whereas in lipid-supplemented cultures very few non-viable cells were detected during the period of the experiment (figure 4-1). By 8-10 hr after cessation of growth, lipid-depleted cultures contained 30-40 per cent non-viable cells.

The effect of anaerobic growth in the absence of lipid supplements on the fatty acid and sterol content of the cells is shown in figure 4-2. It can be seen that cessation of cell division coincided with depletion of unsaturated fatty acids to less than 10 µg/mg dry wt, compared with about 50 µg/mg dry wt. in aerobically-grown cells and 30 µg/mg dry wt in lipid-supplemented anaerobes (see Chapter V). A comparison of the saturated and unsaturated fatty acid content of the cells shows that there were marked changes in the ratio of these during anaerobic growth. Reduction in sterol content of the anaerobically-grown cells followed a similar dilution pattern to that observed for unsaturated fatty acid.

Figure 4-1



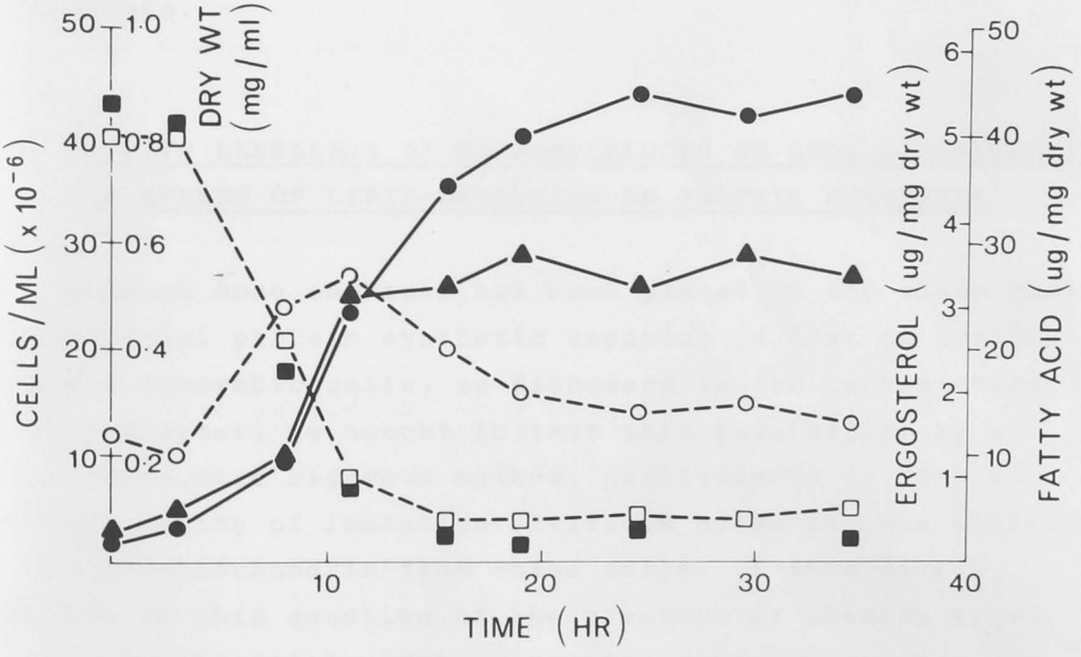
Anaerobic growth of S. cerevisiae.

Wild-type cells were used to inoculate yeast extract/ salts media containing 4 per cent galactose, with and without lipid supplements, to an initial cell density of 0.03 mg/ml. Cells were removed via a syringe.

Lipid-supplemented culture : △—△ , dry wt; ○—○ , cell number; ■—■ , viability.

Lipid-depleted culture : ▲—▲ , cell number; ●—● dry weight; □—□ , viability.

figure 4-2



Anaerobic growth of S. cerevisiae, wild-type, without lipid supplements on yeast extract/salts media.

Legend : ●—● , dry wt; ▲—▲ , cell number;
○—○ , saturated fatty acid; □—□ , ergosterol;
■—■ , unsaturated fatty acid.

In contrast, there were only small changes in fatty acid levels (total and unsaturated) during anaerobic growth of lipid-supplemented cells, although there was a decline in sterol content to about one half that found in aerobic cells. The latter effect is not due to growth limitation by insufficient sterol in the medium and presumably is a consequence of a diminished requirement for sterol under anaerobic conditions.

Because of the loss of viability on extended growth of lipid-poor anaerobic cultures, it was important that the growth period of these cultures be restricted to 22 hr or less. This has been taken into account in the following experiments.

2. *IN VIVO* LABELLING OF MACROMOLECULES OF CELL COMPARTMENTS. THE EFFECT OF LIPID-DEPLETION ON PROTEIN SYNTHESIS

Because some emphasis has been placed on the claim that mitochondrial protein synthetic capacity is lost in lipid-depleted anaerobic cells, as discussed in the introduction to this chapter, we sought to test this possibility by an alternative,more rigorous method, particularly in view of the possibility of isolation artifacts affecting the activity of the promitochondria from these cells. A more direct approach to this question of the presence or absence of an active mitochondrial protein-synthesising system is provided by the *in vivo* method for the assay of this activity described by Schatz and Saltzgaber (1969a). Briefly, this method involves the *in vivo* labelling of cells with ^{3}H-leucine in the presence of cycloheximide (to inhibit cytoplasmic protein synthesis), with and without erythromycin. Under these conditions chloramphenicol or erythromycin-sensitive labelling can be attributed to the mitochondrial protein-synthesising system. The design of the experiment, and the effects of the antibiotics used, are illustrated in Table 4-1. In this experiment aerobically-grown cells

TABLE 4-1

LABELLING OF MITOCHONDRIA *IN VIVO* WITH ^{3}H-LEUCINE

Treatment * (antibiotic addition)	cpm/mg protein mito.	sol.	mitochondrial labelling (% of control)
none	102,000	61,500	100
erythromycin	75,000	55,000	74
chloramphenicol	67,200	49,400	66
cycloheximide (CYC)	9,450	132	100
CYC + erythromycin	567	55	6
CYC + antimycin A	7,200	153	76
CYC + ethidium bromide	2,260	119	24

* Aerobic wild-type cells, growing exponentially on 1 per cent galactose medium, were harvested, washed with water then 0.05 M phosphate (pH 6.0). Washed cells were labelled with ^{3}H-leucine under a stream of nitrogen. The preincubation period was 15 min: labelling was commenced by the addition of 50 µCi of ^{3}H-leucine (34 Ci/mmole) and terminated after 15 min at 29°. Mitochondrial and soluble fractions were prepared after cell breakage via the mechanical method. Where present, antibiotics were added at the following concentrations: erythromycin, 5 mg/ml; chloramphenicol, 4 mg/ml; cycloheximide, 25 µg/ml; antimycin A, 5 µg/ml; ethidium bromide, 50 µg/ml.

were labelled *in vivo* in the presence of the antibiotics indicated, and mitochondrial and soluble fractions prepared from cells disrupted by the mechanical method. In the absence of cycloheximide, both fractions were highly labelled; the specific activity of the mitochondrial fraction was almost twice that of the soluble fraction. Erythromycin or chloramphenicol inhibited the labelling of mitochondrial protein by about 30 per cent. However, cycloheximide gave practically complete inhibition of the incorporation into the soluble fraction, and the mitochondrial incorporation was inhibited nearly 93 per cent. In the presence of cycloheximide the specific activity of the mitochondrial fraction was approximately 70 times that of the soluble fraction (cf. relative labelling in absence of cycloheximide) and this mitochondrial labelling was almost completely inhibited by erythromycin. Antimycin A, at a concentration sufficient to inhibit respiration by greater than 95 per cent, caused a relatively small inhibition of mitochondrial incorporation indicating a large degree of independence of incorporation from respiration (as would be expected, as the incorporation was carried out under nitrogen). The effect of ethidium bromide is discussed later in this chapter. The lipid content of the cells used was 58 mg unsaturated fatty acid per gm dry wt (83 per cent of total fatty acid was unsaturated) and 5.5 mg ergosterol per gm dry wt: these values are typical of highly aerobic cells, and can be compared with other cell types, presented later.

It appears that mitochondria can be labelled in the presence of cycloheximide, and under these conditions the process is highly selective. The sensitivity of this labelling to inhibitors such as erythromycin indicates that the incorporation is a consequence of an active mitochondrial protein-synthesising system. These results agree closely with those presented by Schatz and Saltzgaber (1969a) and by Schweyen and Kaudewitz (1970).

The incorporation levels presented in Table 4-1 also provide an estimate of the relative activity of mitochondrial

and cytoplasmic protein-synthesising systems in aerobic cells. Firstly, assuming that mitochondrial protein accounts for about 10 per cent of total cell protein, the total labelling of mitochondrial protein in the absence of antibiotics is 20 per cent or less of total cell labelling. If it is assumed that the cyloheximide-insensitive, erythromycin-sensitive labelling represents the activity of the mitochondrial protein-synthesising system, then this accounts for less than 10 per cent of the labelling of mitochondrial protein in the absence of antibiotics, i.e. the activity of the mitochondrial system accounts for less than 2 per cent of the total cell protein synthetic activity, and whole cell labelling without antibiotics can be attributed to the activity of the cytoplasmic protein-synthesising system.

In the experiments presented below on promitochondrial labelling the above selective-labelling procedure (in the presence of cycloheximide) has been used. The promitochondrial fractions have been defined both operationally (10,000 g sediment, band at density of 1.15-1.20 in sucrose density gradient) and enzymically (presence of oligomycin-sensitive ATPase). Oligomycin-sensitive ATPase was used as a marker for promitochondria as this is one of the few enzymes which is characteristic of mitochondria, yet is still present in anaerobic cells (Schatz, 1963; Criddle and Schatz, 1969).

Promitochondria prepared from lipid-depleted anaerobes accounted for 50-60 per cent of the oligomycin-sensitive ATPase found in the cell-free homogenate (Table 4-2). After pulse-labelling *in vivo* in the presence of cycloheximide (as described above and in Chapter II) there was little incorporation of isotope into promitochondrial protein from this type of cell (Table 4-2). There was no significant effect of erythromycin on this labelling. Promitochondria from lipid-supplemented anaerobes also accounted for the major part of the oligomycin-sensitive ATPase of the cell homogenate (Table 4-2). However, in contrast to the results obtained with the lipid-depleted promitochondria,

TABLE 4-2

PROTEIN SYNTHETIC ACTIVITY OF ANAEROBICALLY-GROWN CELLS

Anaerobe	ATPase[2]	Incorporation[1]					
		PROMITOCHONDRIA		SOLUBLE		CELLS	
		-ER	+ER	-ER	+ER	-CYC	+CYC
Lipid Supplemented	70(60)	2280	140	182	141	2130	43
Lipid depleted	29(56)	72	30	112	52	40	37

Cells were grown anaerobically with or without lipid supplements for 20 hr then labelled *in vivo* using two procedures. The first was the procedure for selective labelling of the promitochondrial fraction, described in the text. The second was the routine whole cell labelling described in Chapter II (section 13 a).

1. Promitochondrial and soluble protein labelling was carried out in the presence of cycloheximide (10 µg/ml), with or without erythromycin(ER, 5 mg/ml); values are cpm/mg protein. Whole cell labelling was carried out in the presence or absence of cycloheximide (CYC, 10 µg/ml); values are cpm/10 min/mg cells.

2. ATPase activity (of the promitochondrial fraction) is expressed as µg Pi released/min/mg protein, and is 90-95% sensitive to 50 µg oligomycin/mg protein. Values in parentheses are per cent of whole cell homogenate activity found in the promitochondrial fraction.

labelling *in vivo* of promitochondria was quite substantial; this labelling was almost completely sensitive to erythromycin. In the presence of cycloheximide there was little incorporation into the soluble fraction of either cell type, and this residual labelling was little affected by erythromycin.

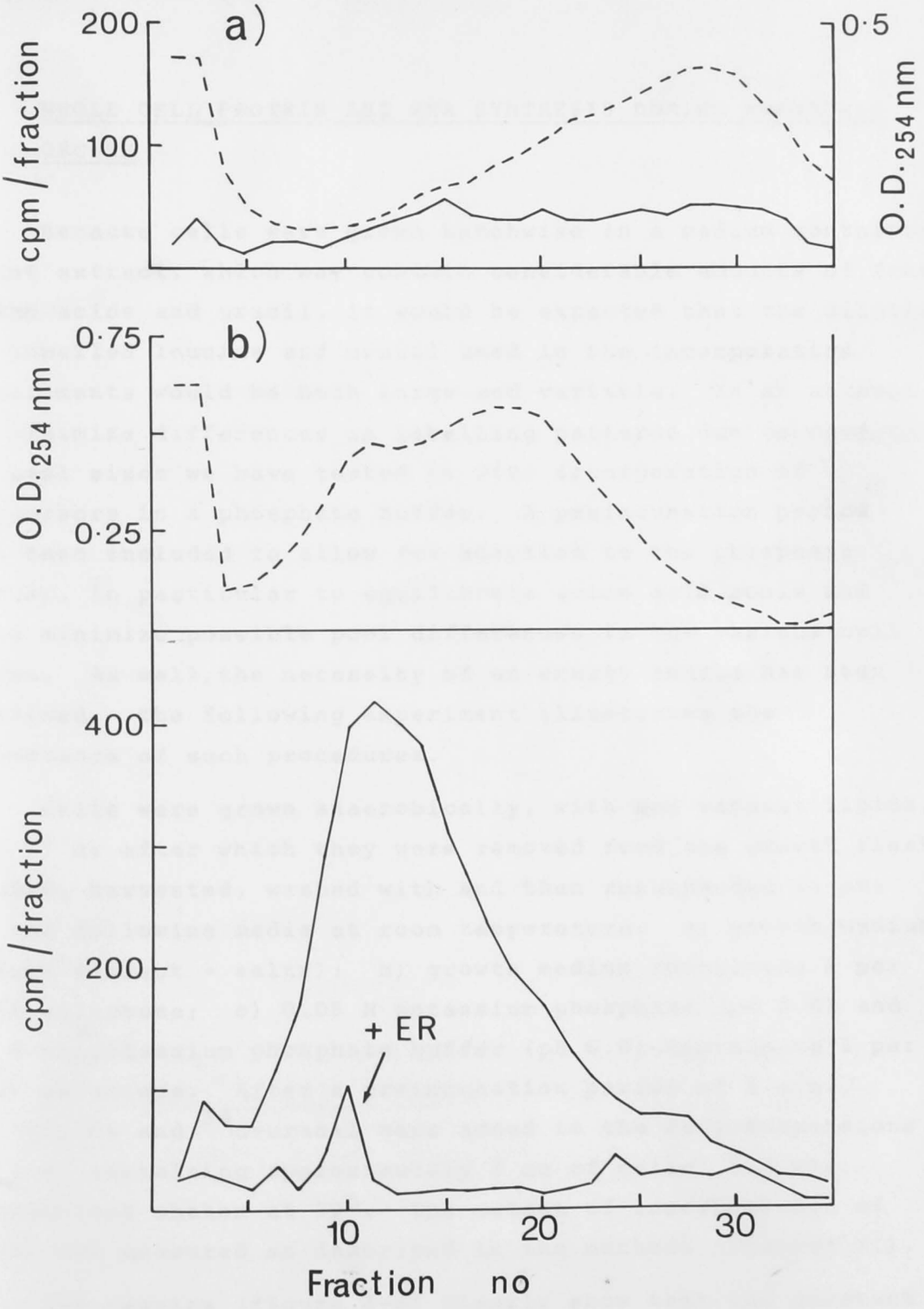
The sedimentation behaviour of the promitochondrial fractions on sucrose density gradients, and the distribution of associated label, is shown in figures 4-3a, b. Clearly, the labelling is associated with promitochondria in lipid-supplemented cells, and erythromycin eliminates this labelling. Promitochondria from lipid-depleted cells were not labelled under either condition.

However, even in the absence of cycloheximide, there was little, if any, labelling of the whole cell protein of lipid-depleted anaerobically-grown cells, whereas in lipid-supplemented cells or aerobic cells pulsed under the same conditions there was extensive labelling (Table 4-2). This failure to incorporate significant amounts of label into cellular protein in the absence of antibiotics could be due to either a very large increase in the pool size of leucine in lipid-depleted cells during anaerobic growth, or to a very severely restricted capacity to synthesise protein. Measurement of the leucine content of the free amino acid of the two anaerobic cell types gave values of 0.2-0.5 and 0.5-0.9 µmoles leucine/100 mg dry wt (3 experiments) for lipid-depleted and supplemented anaerobes respectively. It is thus unlikely that the leucine pool size of the cells accounts for the apparent incapacity of lipid-depleted cells to incorporate ^{3}H-leucine into protein.

From the above results it appeared that neither the promitochondrial nor the cytoplasmic fraction were able to incorporate ^{3}H-leucine into protein, i.e. the capacity to synthesise proteins had been lost by both cell compartments during anaerobic growth in the absence of lipid supplements. To determine the kinetics of this loss the capacity of cells to incorporate ^{3}H-leucine into whole-cell protein at different

figure 4-3

Promitochondria from cells grown anaerobically with and without lipid supplements : distribution of label on sucrose density gradients. The promitochondria were labelled with ^{3}H-leucine, and fractionated, as described in Chapter II. Fig (a) shows the patterns of promitochondria from lipid-depleted cells, while fig (b) shows patterns obtained from the promitochondria of lipid-supplemented cells. Broken lines indicate the optical density traces : solid lines indicate radioactivity. Fig (b) shows the labelling of lipid-supplemented promitochondria in the presence and absence of erythromycin (ER, 5 mg/ml).


a)
200
100
cpm / fraction
0·5
O.D.254 nm
b)
0·75
0·25
O.D.254 nm
400
200
cpm / fraction
+ ER
10
20
30
Fraction no

times during anaerobic growth was measured. As well, RNA labelling was followed to provide a comparison with the protein labelling pattern.

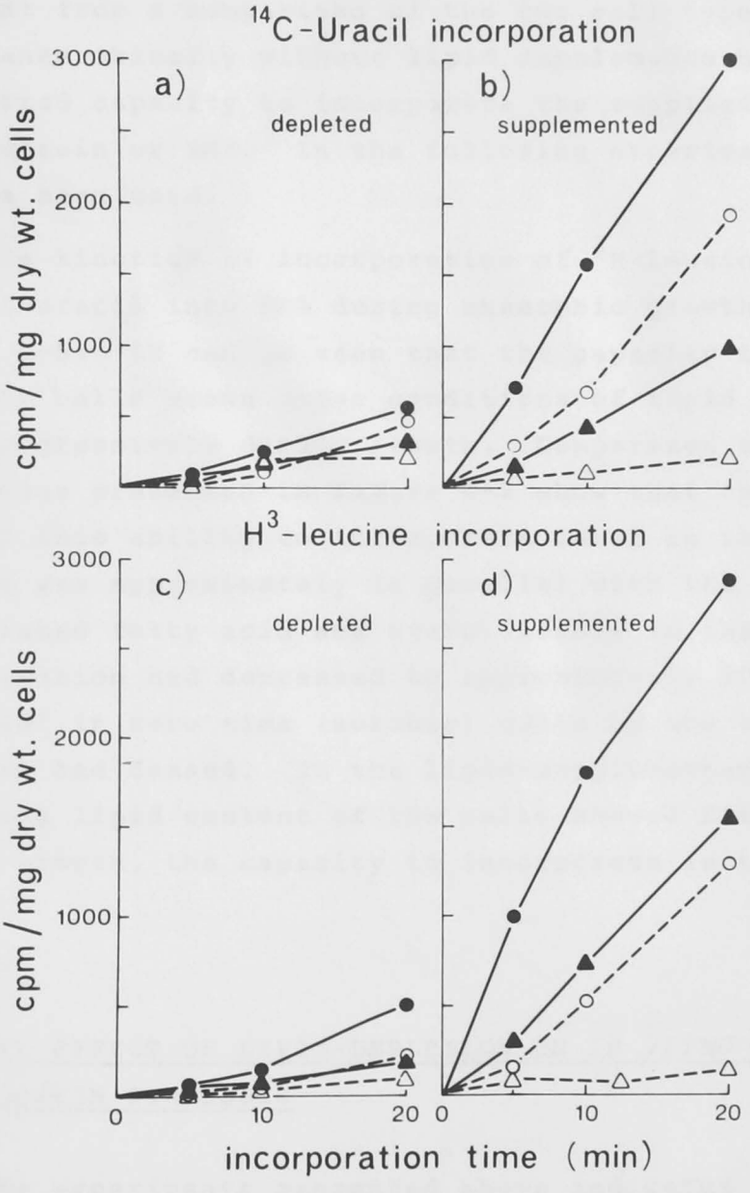
3. WHOLE CELL PROTEIN AND RNA SYNTHESIS DURING ANAEROBIC GROWTH

Because cells were grown batchwise in a medium containing yeast extract, which may contain considerable amounts of free amino acids and uracil, it would be expected that the dilution of labelled leucine and uracil used in the incorporation experiments would be both large and variable. In an attempt to minimize differences in labelling patterns due to changes in pool sizes we have tested *in vivo* incorporation of precursors in a phosphate buffer. A preincubation period has been included to allow for adaption to the phosphate buffer, in particular to equilibrate amino acid pools and thus minimize possible pool differences in the various cell types. As well, the necessity of an energy source has been examined. The following experiment illustrates the importance of such procedures.

Cells were grown anaerobically, with and without lipids, for 17 hr after which they were removed from the growth flask, cooled, harvested, washed with and then resuspended in one of the following media at room temperature: a) growth medium (yeast extract - salts); b) growth medium containing 1 per cent galactose; c) 0.05 M potassium phosphate (pH 6.0) and d) 0.05 $\frac{M}{\lambda}$ potassium phosphate buffer (pH 6.0) containing 1 per cent galactose. After a preincubation period of 5 min. ^{3}H-leucine and ^{14}C-uracil were added to the cell suspensions (1.5 ml containing approximately 5 mg of cells) and the suspensions shaken at 29^{o}. The extent of incorporation of label was measured as described in the methods (Chapter II).

The results (figure 4-4) clearly show that the greatest incorporation, for both cell types and both labelled precursors, is achieved in a phosphate medium, and that the

Figure 4-4



Incorporation of ^{14}C-uracil and ^{3}H-leucine by anaerobically-grown cells. The type of anaerobic growth (with or without lipid supplementation) is indicated. The different symbols define the incorporation media, as described in the text : ●—● , phosphate buffer containing galactose; ▲—▲ , phosphate buffer; ○—○ , growth medium containing galactose; △—△ , growth medium.

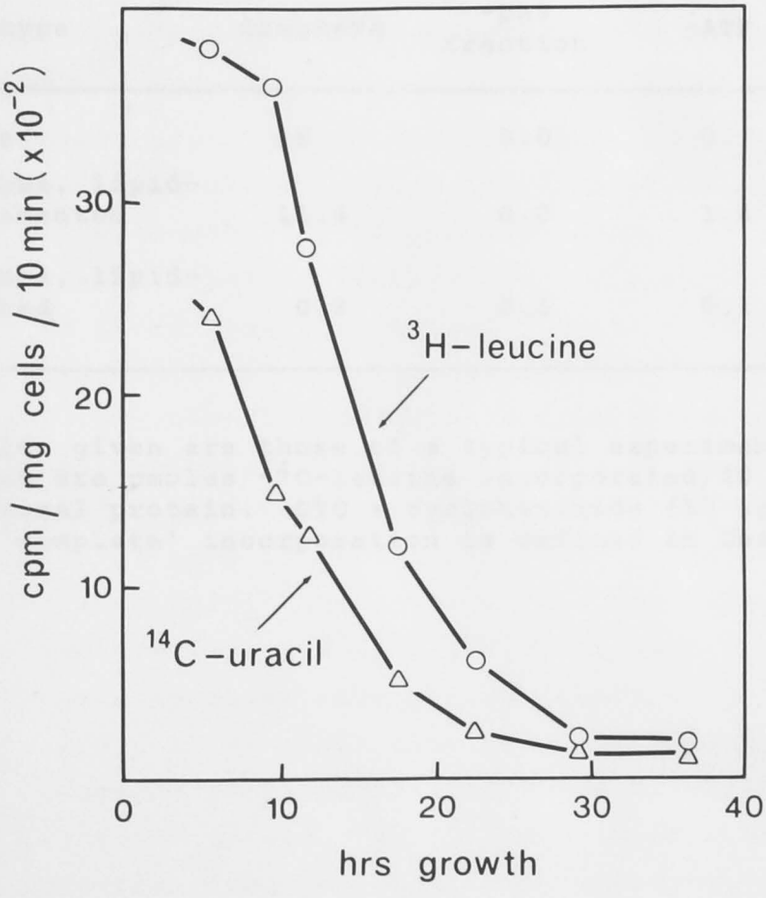
incorporation is very dependent on the presence of galactose, presumably as an energy source. It is also apparent from a comparison of the two cell types that cells grown anaerobically without lipid supplements have a very restricted capacity to incorporate the supplied precursors into protein or RNA. In the following experiments medium (d) has been used.

The kinetics of incorporation of ^{3}H-leucine into protein and ^{14}C-uracil into RNA during anaerobic growth are shown in figure 4-5. It can be seen that the capacity to incorporate label by cells grown under conditions of lipid limitation fell progressively during growth. Comparison of these results with those presented in figure 4-2 show that the progressive fall of this ability to incorporate label in the lipid-limited culture was approximately in parallel with the decrease in unsaturated fatty acid and sterol levels in these cells. Incorporation had decreased to approximately 30 per cent of the level in zero time (aerobic) cells by the time cell division had ceased. In the lipid-supplemented culture, where the lipid content of the cells showed little change during growth, the capacity to incorporate isotope remained high.

4. THE EFFECT OF LIPID-DEPLETION ON *IN VITRO* CYTOPLASMIC PROTEIN SYNTHESIS

The experiments presented above indicated that lipid starvation during anaerobic growth led to the loss of the ability to synthesise proteins, and that this was true of both the cytoplasmic and the mitochondrial protein-synthesising systems. There are several possible reasons for this loss. As shown above, an energy source is required for incorporation, so there may be some failure in this energy supply. Because the lipid composition of the membranes of lipid-depleted cells is radically altered (figure 4-2; Chapter V) it is possible that membrane transport is affected with consequent

Figure 4-5



Incorporation of ^{3}H-leucine and ^{14}C-uracil by wild-type cells after periods of anaerobic growth without lipid supplements. The incorporation procedure is described in the text, and in Chapter II.

TABLE 4-3

AMINO ACID INCORPORATION BY
CYTOPLASMIC RIBOSOMES

Cell type	Complete	-pH5 fraction	-ATP	+CYC
Aerobe	19	0.0	0.1	5.2
Anaerobe, lipid-supplemented	11.4	0.0	1.4	2.4
Anaerobe, lipid-depleted	0.3	0.1	0.1	0.4

Results given are those of a typical experiment. The values are pmoles ^{14}C-leucine incorporated/20 min/mg ribosomal protein. CYC = cycloheximide (50 µg/ml). The 'complete' incorporation is defined in Chapter II.

effects on energy supply and/or amino acid uptake. Alternatively, lipid depletion may cause a failure of the protein-synthetic mechanism itself.

In an attempt to define more precisely the reasons for the loss of protein synthesis, we prepared cytoplasmic ribosome and supernatant fractions from lipid-depleted and lipid-supplemented cells and tested the ability of reconstituted systems to incorporate amino acids into protein. The results of these experiments are shown in Table 4-3.

Systems reconstituted from aerobically-grown cells or cells grown anaerobically with lipid supplements are both active. The sensitivity to cycloheximide, and the dependence on both the supernatant fraction and an energy source, are similar to those described by Lamb, Clark-Walker, and Linnane (1968). Incorporation was linear for at least 20 min. In contrast, the reconstituted system from cells grown anaerobically without lipids had little or no activity. This suggests that the inactivity *in vivo* is not a consequence of lack of ATP, nor of failure to take up the labelled amino acid. The possibility that ribosomal or soluble factors were inactive in the reconstituted system were examined by cross-mixing experiments. These are shown in Table 4-4. It is clear that both the supernatant fraction and the ribosome fraction were affected by lipid-depletion during anaerobic growth. Thus, when either ribosomes or soluble fraction from depleted cells were tested with the complementary fraction from the active lipid-supplemented cell, the reconstituted system had only low endogenous activity and was at best partially stimulated by the addition of RNA. Some activity was observed in the mixture of ribosomes from supplemented cells with the supernatant fraction from depleted cells. This may indicate that the ribosomes are primarily affected by lipid depletion, and that loss of activity of the supernatant factor(s) is a consequence of turnover of these components. The systems containing both ribosomal and soluble fractions from the

TABLE 4-4

TEST FOR ACTIVITY OF RIBOSOME AND pH5 FRACTIONS

Source[1]				
Ribosome	pH5 fraction	+RNA[2]	-RNA	+CYC[3] (+RNA)
Supp.	Supp.	24.1	5.1	0.9
Depl.	Depl.	0.7	0.8	0.6
Supp.	Depl.	6.8	1.6	1.0
Depl.	Supp.	1.8	1.3	1.0

Results given are those of a typical experiment in which cells were grown anaerobically, with and without lipid supplements, for 17 hr. Ribosome and pH5 fractions were prepared from both cell types, and then tested in the *in vitro* system for activity.

Values given are as in Table 4-3.

1. These columns give source of fraction, Supp. = from lipid-supplemented cells; Depl. = from lipid-depleted cells.

2. Whole cell yeast RNA was added (50 µg/ml).

3. Concentration of cycloheximide (CYC) was 50 µg/ml.

lipid-depleted cells were only fractionally activated by the addition of high-molecular weight RNA, or by poly U when ^{14}C-phenylalanine was used as a label.

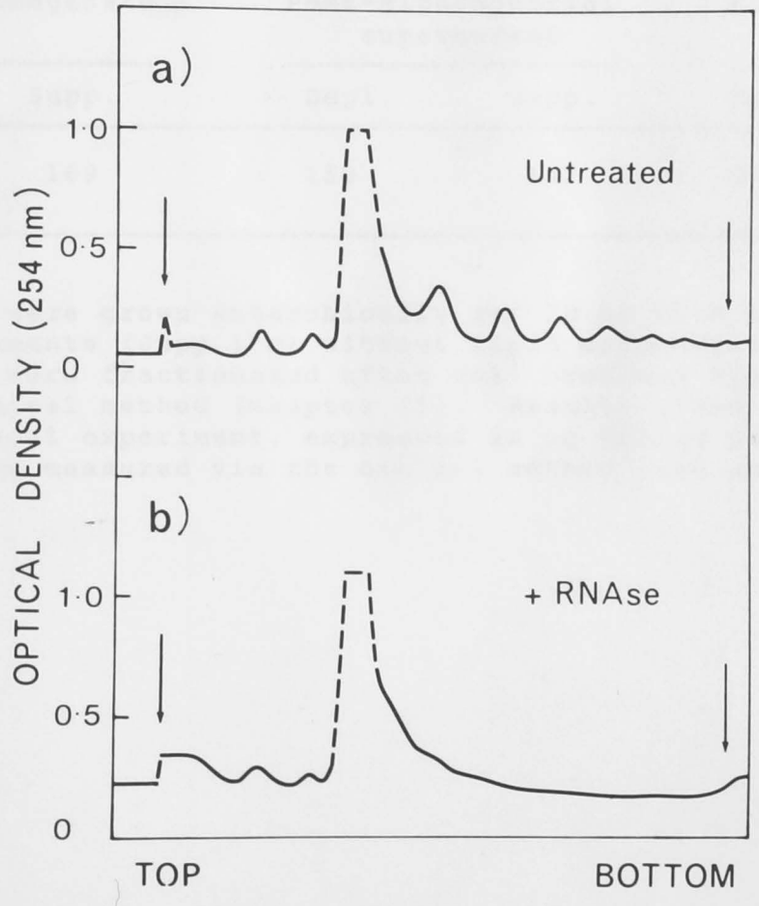
The lack of stimulation by added RNA in the case of the *in vitro* system from lipid-depleted cells would suggest that the inactivity of these cells is not simply due to lack of messenger RNA. This is supported by analysis of the ribosomal fractions on linear sucrose density gradients (figure 4-6). Ribosome preparations from cells grown anaerobically for 17 hr in the absence of lipid supplements contained the same proportion (approximately 30 per cent) of polysomes as cells grown with lipid supplements for the same time. In this respect, both anaerobic cell types were similar to exponential phase cells cultured aerobically.

5. WHOLE CELL RNA CONTENT AFTER ANAEROBIC GROWTH

In view of the loss of RNA synthetic capacity during anaerobic growth without lipid supplements, shown earlier, we have examined the effect of anaerobic growth on the whole cell RNA content and the distribution of RNA species. Table 4-5 shows the total RNA content of cell fractions prepared from cells grown anaerobically with and without lipids for 17 hr. There was no marked difference in the RNA content, expressed on a protein basis, between corresponding fractions. This is also true of the RNA/dry weight ratios for whole cells.

The ribosome fraction and whole cell homogenate were further analysed: the RNA was extracted and then fractionated using polyacrylamide gel electrophoresis (Loening, 1969). The electrophoretic patterns of the RNA from the two anaerobic cell types are shown in figure 4-7. There is no obvious difference in the number of species of RNA, or of their relative proportions, with the possible exception of 4S and 5S RNA species. The 18S and 25S RNA species of the cytoplasmic ribosomes are present in the

Figure 4-6



Polyribosomes from lipid-depleted anaerobically-grown cells, and the effect of RNAse. The ribosome fraction from depleted cells was loaded onto a linear sucrose gradient (described in Ch. II) and run for 2½ hr at 25,000 rpm. In some cases, RNAse (50 µg/ml) was added to the ribosome fraction just prior to centrifugation. The gradients were analysed on an ISCO fractionator; the arrows mark the top and bottom of the tubes.

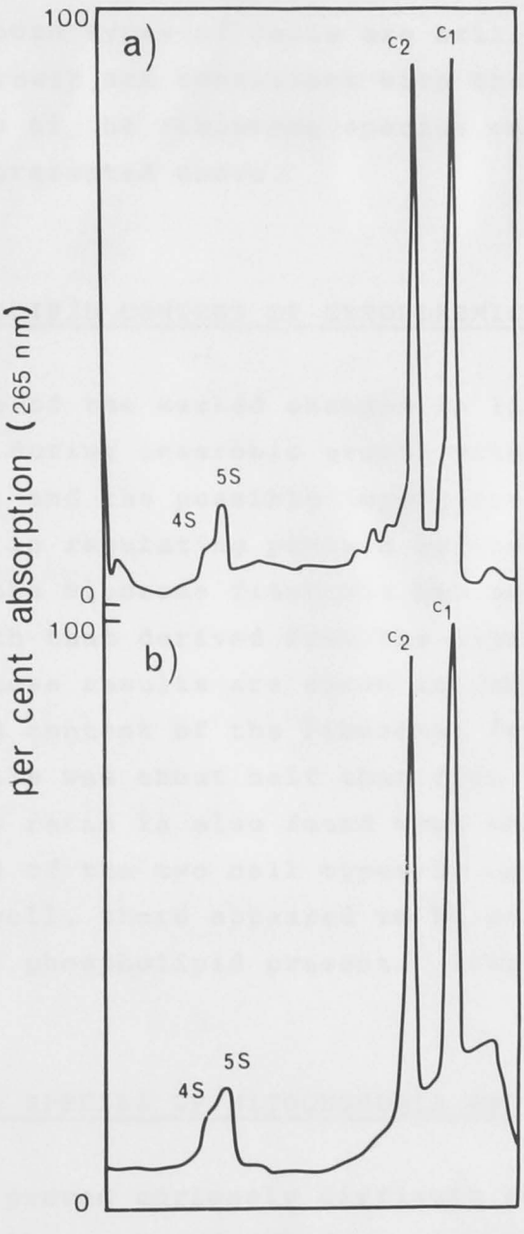
TABLE 4-5

RNA CONTENT OF CELL FRACTIONS FROM DEPLETED AND SUPPLEMENTED CELLS

Whole Homogenate		Post-Mitochondrial supernatant		Ribosomes	
Depl.	Supp.	Depl.	Supp.	Depl.	Supp.
161	169	159	142	571	530

Cells were grown anaerobically for 20 hr with lipid supplements (Supp.) or without lipid supplements (Depl.). Cells were fractionated after cell breakage via the mechanical method (Chapter II). Results given are of a typical experiment, expressed as µg RNA/mg protein. RNA was measured via the orcinol method (see appendix).

Figure 4-7



The RNA species from ribosomes of cells grown anaerobically (a) without or (b) with lipid supplements. Extraction and electrophoresis conditions are described in Chapter II. Gels were run for 2 hr at room temperature. The cytoplasmic ribosomal RNA species are denoted C_1 (25S) and C_2 (18S).

expected ratio and most importantly, there is no sign of degradation. These results, which indicate that the RNA species of both types of cells are still intact after anaerobic growth,are consistent with those obtained from the analysis of the ribosomal species on sucrose density gradients, presented above.

6. PHOSPHOLIPID CONTENT OF CYTOPLASMIC RIBOSOMES

Because of the marked changes in lipid content of the whole cells during anaerobic growth without lipid-supplements, and the possible importance of membrane-ribosome interaction in regulating protein synthesis, the phospholipid content of the ribosome fractions has been examined and compared with that derived from the lipid-supplemented culture. These results are shown in Table 4-6. The phospholipid content of the ribosomal fraction from lipid-depleted cells was about half that from lipid-supplemented cells. This ratio is also found when whole cell phospholipid of the two cell types is compared (Jollow *et al.* 1968). As well, there appeared to be marked differences in the types of phospholipid present. (Table 4-6).

7. THE RNA SPECIES OF MITOCHONDRIA AND PROMITOCHONDRIA

It has proved curiously difficult to isolate unique species of ribosomes and RNA from mitochondria of *S. cerevisiae*. Almost certainly the main difficulty has been purification of the mitochondria from cytoplasmic ribosomes. In this respect it has been suggested that there is a functional interaction of cytoplasmic ribosomes with mitochondria (Schmitt, 1970). The purification problem can be readily appreciated by an examination of figure 4-8, which shows a thin section of *S. cerevisiae* grown aerobically in which the cell wall has been largely digested away by snail gut enzyme to facilitate fixing and staining. The fixing/staining

TABLE 4-6

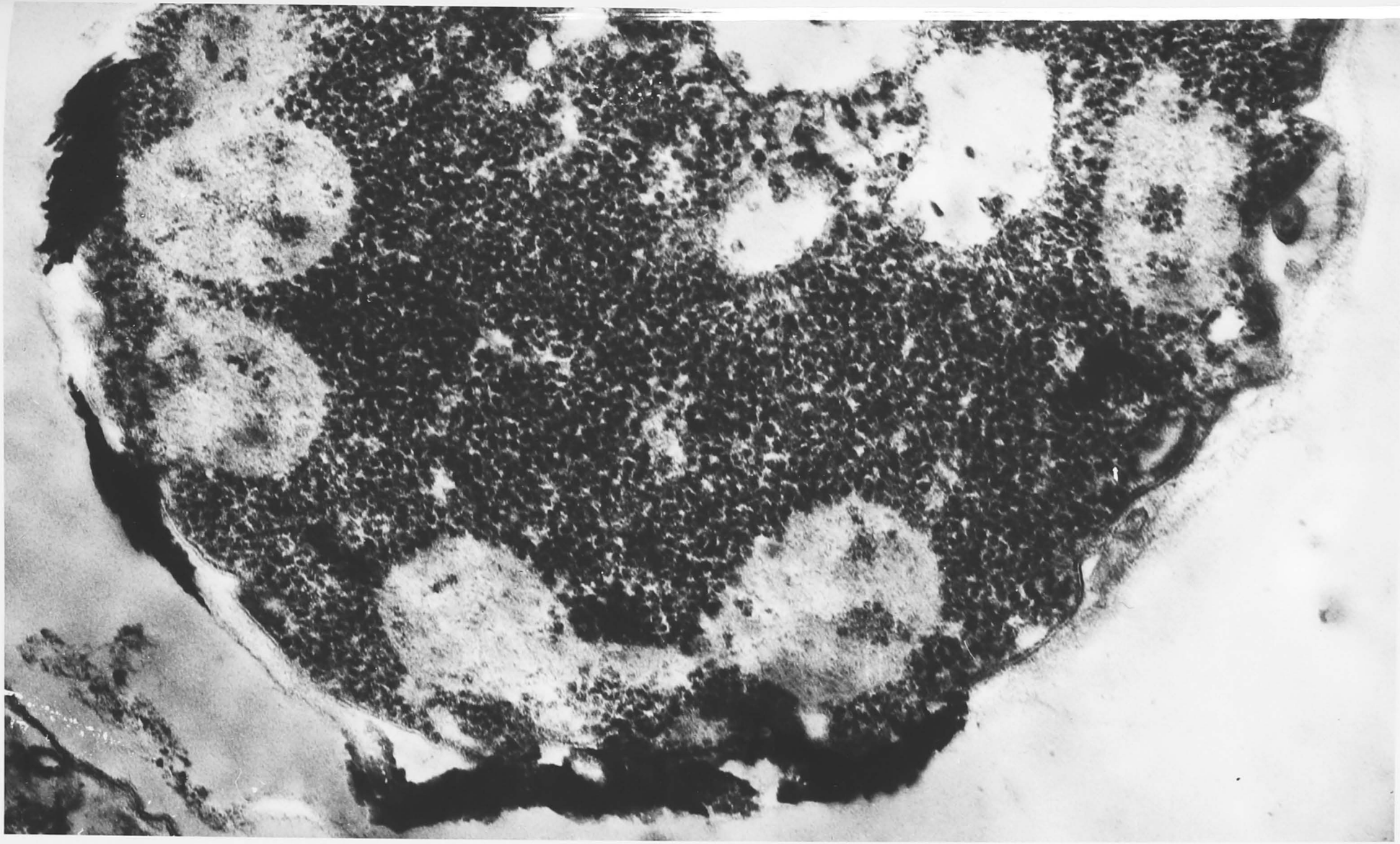
PHOSPHOLIPIDS OF RIBOSOMES FROM DEPLETED AND SUPPLEMENTED CELLS

Growth Conditions	Total Phospholipid		Distribution (%w/w)			
	mg/mg protein	mg/mg RNA	PE	PC	PI	PS
Lipid-Depleted	31	55	33.8	52.6	1.2	12.4
Lipid-Supplemented	63	120	31.6	30.4	5.0	33.0

Cells were grown anaerobically for 20 hr with and without lipid supplements. Ribosomes were prepared as described in Chapter II after cell breakage via the mechanical method. Results are of a typical experiment.

Phospholipid classes examined are phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), phosphatidyl inositol (PI), and phosphatidyl serine (PS).

figure 4-8. Thin section through an aerobically-grown *S. cerevisiae* cell. Enlargement is approximately 60,000 times. Experimental details are given in the text and in Chapter II.



procedures used, glutaraldehyde then osmium tetroxide, favour the visualization of ribosomes : the mitochondria are poorly defined under these conditions. Nevertheless, the relatively clear areas on the periphery of the cell, by reference to permanganate-stained cells, undoubtedly represent mitochondria. The abundance of cytoplasmic ribosomes, and particularly their excess over what appear to be mitochondrial ribosomes, emphasises the purification problem.

Even after the washed mitochondrial fraction from aerobically-grown cells, prepared by differential centrifugation after cell rupture via the mechanical method, had been purified by banding in a discontinuous gradient and pelleting though 30 and 40 per cent sucrose, it was found that very little other than cytoplasmic ribosomal RNA could be detected on polyacrylamide gels. However, recovery of unique species of high molecular weight RNA could be achieved if cells were pre-fixed with glutaraldehyde prior to cell breakage. Presumably, glutaraldehyde stabilizes the mitochondrial membranes, so that the fractions prepared are more discrete.

Typical gel traces of the RNA species extracted from the mitochondrial or promitochondrial fractions, purified as above, are shown in figure 4-9: the fractions were prepared from cells grown aerobically on 1 per cent galactose (figure 4-9,a), anaerobically with lipid supplements (figure 4-9,b), and anaerobically without lipids. The RNA species from cytoplasmic ribosomes (denoted C_1 and C_2) were present in all traces. Mitochondrial RNA species could only be detected in RNA extracted from the (pro)mitochondrial fractions of aerobically-grown cells or cells grown anaerobically <u>with</u> lipid supplements. This failure to recover RNA from lipid-depleted promitochondria may indicate its absence from these organelles. On the other hand, because of the gross change of membrane composition of these organelles, the possibility cannot be excluded that the RNA is lost during preparation and purification of the

figure 4-9

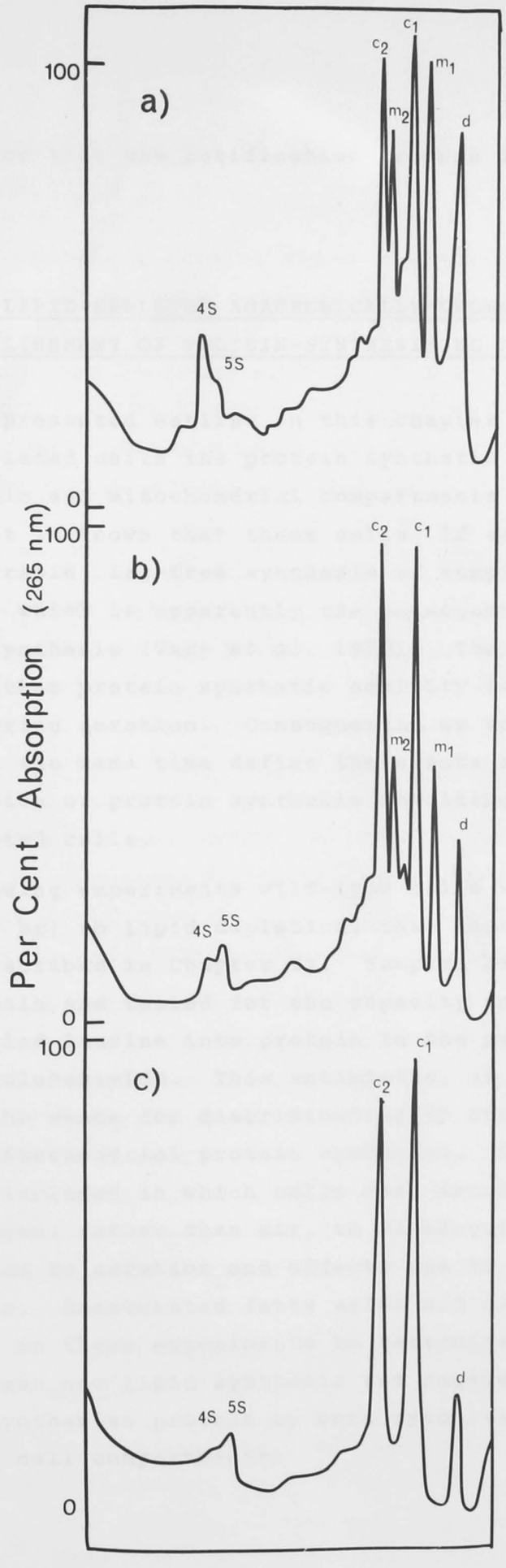
RNA species from (pro)mitochondria of *S. cerevisiae*. After growth, cells were harvested and washed, then incubated with glutaraldehyde (final concentration 2.5%) for 30 min at 0°C. Cells were washed, broken by the mechanical method, then a mitochondrial fraction (1000g - 10,000g sediment) prepared. This fraction, twice washed, was banded in a discontinuous gradient, the band collected and diluted to approximately 0.5M sucrose, and layered onto a 2-step sucrose gradient (30 and 40 per cent). These gradients were centrifuged for 1 hr at 27,000 rpm in a SW 27.1 head: the mitochondrial pellet was extracted for RNA as detailed in Chapter II. Gels were run for 2 hr at room temperature. The gel traces above show the RNA species isolated from (pro)mitochondria from :

a) aerobically-grown cells;

b) anaerobic, lipid-supplemented cells;

c) anaerobic, lipid-depleted cells.

Cytoplasmic ribosomal RNA species are designated C_1 and C_2, as in fig 4-7; the mitochondrial ribosomal species are designated M_1 and M_2. The peak denoted D is DNAse-sensitive and RNAse-resistant. Traces a) and b) were obtained by R. Yu.


Per Cent Absorption (265 nm)
100
0
a)
c_2
c_1
m_1
m_2
d
4S
5S
100
0
b)
c_2
c_1
m_2
m_1
d
4S
5S
100
0
c)
c_2
c_1
d
4S
5S

promitochondria, or that the purification process is incomplete.

8. AERATION OF LIPID-DEPLETED ANAEROBICALLY-GROWN CELLS. THE RE-ESTABLISHMENT OF PROTEIN-SYNTHESISING ACTIVITY

The results presented earlier in this chapter showed that in lipid-depleted cells the protein synthetic activity of both cytoplasmic and mitochondrial compartments was much decreased. Yet it is known that these cells, if aerated, are capable of a rapid, lag-free synthesis of respiratory and other enzymes which is apparently the consequence of *de novo* protein synthesis (Vary *et al.* 1970). The latter finding suggests that protein synthetic activity is re-established during aeration. Consequently we sought to quantitate and at the same time define the events responsible for the reactivation of protein synthesis resulting from aeration of depleted cells.

In the following experiments wild-type cells were grown anaerobically (20 hr) to lipid depletion, then harvested and aerated as described in Chapter II. Samples of cells were removed at intervals and tested for the capacity to incorporate labelled leucine into protein in the presence and absence of cycloheximide. This antibiotic, as indicated above, provides the means for discriminating *in vivo* between cytoplasmic and mitochondrial protein synthesis. Control experiments were included in which cells were incubated under a stream of nitrogen, rather than air, to distinguish between effects due to aeration and effects due to step-up culture conditions. Unsaturated fatty acids and ergosterol were also assayed in these experiments to determine the relationship between new lipid synthesis and regeneration of the capacity to synthesise protein by both cytoplasmic and mitochondrial cell compartments.

(i) Re-establishment of cytoplasmic protein synthesis

Figure 4-10 shows the rate at which whole cells re-established protein synthesis during aeration. As calculated earlier, this activity is due largely to the cytoplasmic protein-synthesising system. Although the kinetics of induction of this activity varied to some extent from experiment to experiment, the increase in incorporation over a 2 hr aeration period was always greater than 10-fold.

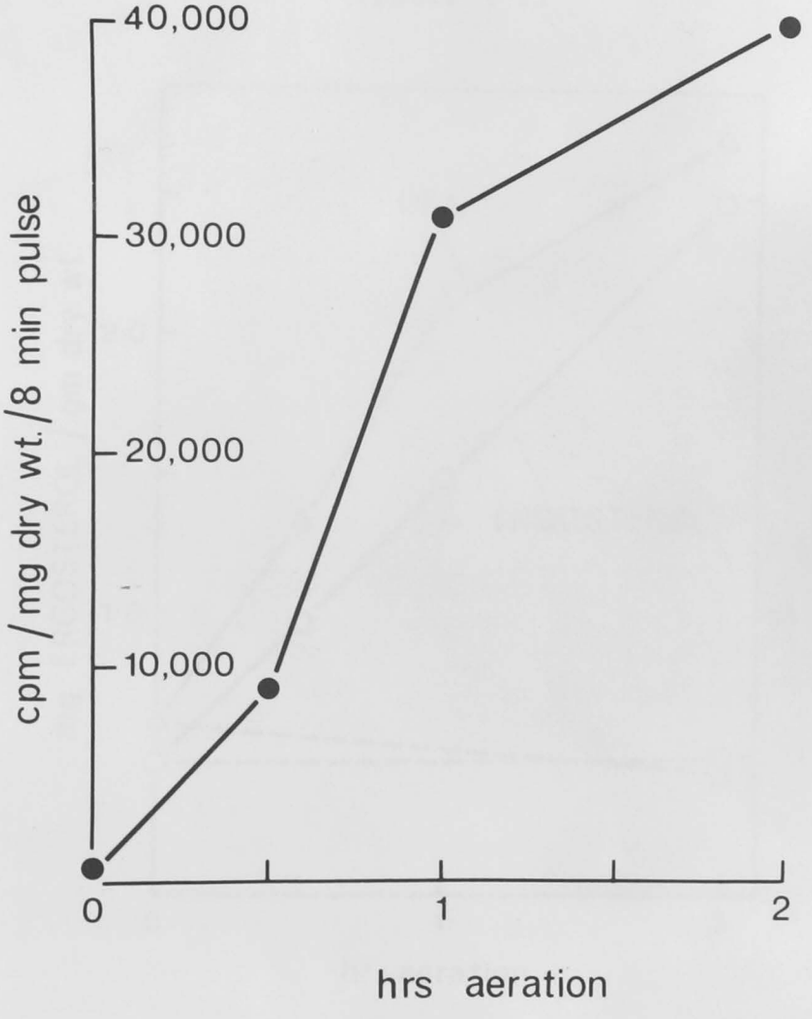
There was also a substantial increase in lipid content of the cells during the aeration period (figure 4-11). Both ergosterol and the unsaturated fatty acid (palmitoleic and oleic acids) content increased several fold in this time. If the incubation was carried out under nitrogen instead of air there was no synthesis of either lipid (figure 4-11). On the other hand the effect on protein synthesis of replacing air with nitrogen during the induction period was surprising. The activity of the cytoplasmic protein-synthesising system in these cells was lower than found in aerated cells, but very much greater in some experiments than in anaerobic cells.

The presence of inhibitors of mitochondrial protein synthesis (erythromycin, ethidium bromide) had little effect on the development of cytoplasmic protein synthesis (figure 4-12). Erythromycin or ethidium bromide, at the concentrations used, had no effect on induced lipid synthesis. It would thus appear that the re-establishment of whole cell protein synthesis does not depend on the activity of the mitochondrial protein-synthesising system.

(ii) Re-establishment of mitochondrial protein synthesis

Mitochondrial protein synthesis was measured by *in vivo* labelling of (pro)mitochondrial protein with ^{3}H-leucine, as described earlier. The results of two such experiments are shown in Table 4-7, together with whole cell lipid analyses.

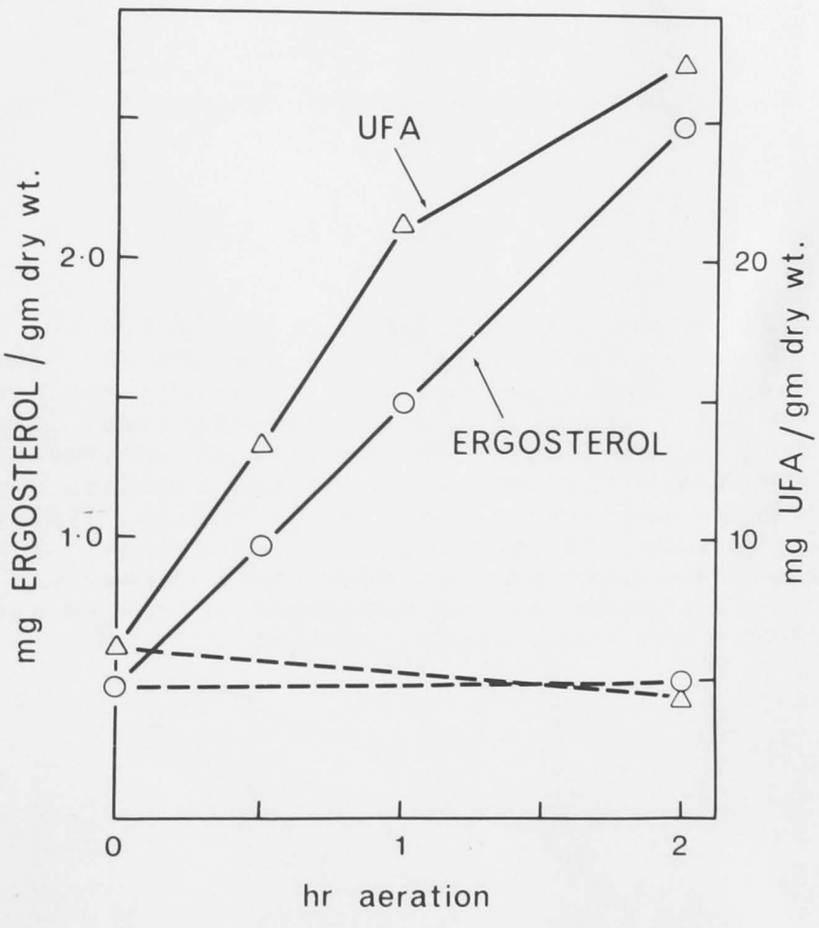
Figure 4-10



Re-establishment of cytoplasmic protein synthesis during aeration of anaerobically-grown, lipid-depleted cells. Incorporation was tested in phosphate/galactose buffer; the labelling period was 8 min (under nitrogen).

The experiment was carried out as detailed in Ch. II, with ^{14}C-leucine (2 µCi/µmole) as precursor; 0.6 µCi was added to approx. 5 mg dry wt cells in 1.5 ml buffer.

Figure 4-11



Synthesis of lipids (ergosterol and unsaturated fatty acids, UFA) during aeration of lipid-depleted, anaerobically-grown cells. Dotted lines indicate that the incubation was carried out under nitrogen, rather than air.

figure 4-12

Time course of incorporation of ^{3}H-leucine by cells grown anaerobically, without lipid supplements, then aerated for 4 hr under various conditions. Control cells were incubated under air or nitrogen, as indicated. Other cultures were aerated in the presence of antibiotics. Where present, antibiotics were added to the aeration medium at the following concentrations: erythromycin, 5 mg/ml; ethidium bromide, 50 µg/ml; and cycloheximide, 25 µg/ml. After the 'aeration' period, cells were washed and their incorporation tested in the phosphate/galactose medium, as described in the text, under nitrogen.

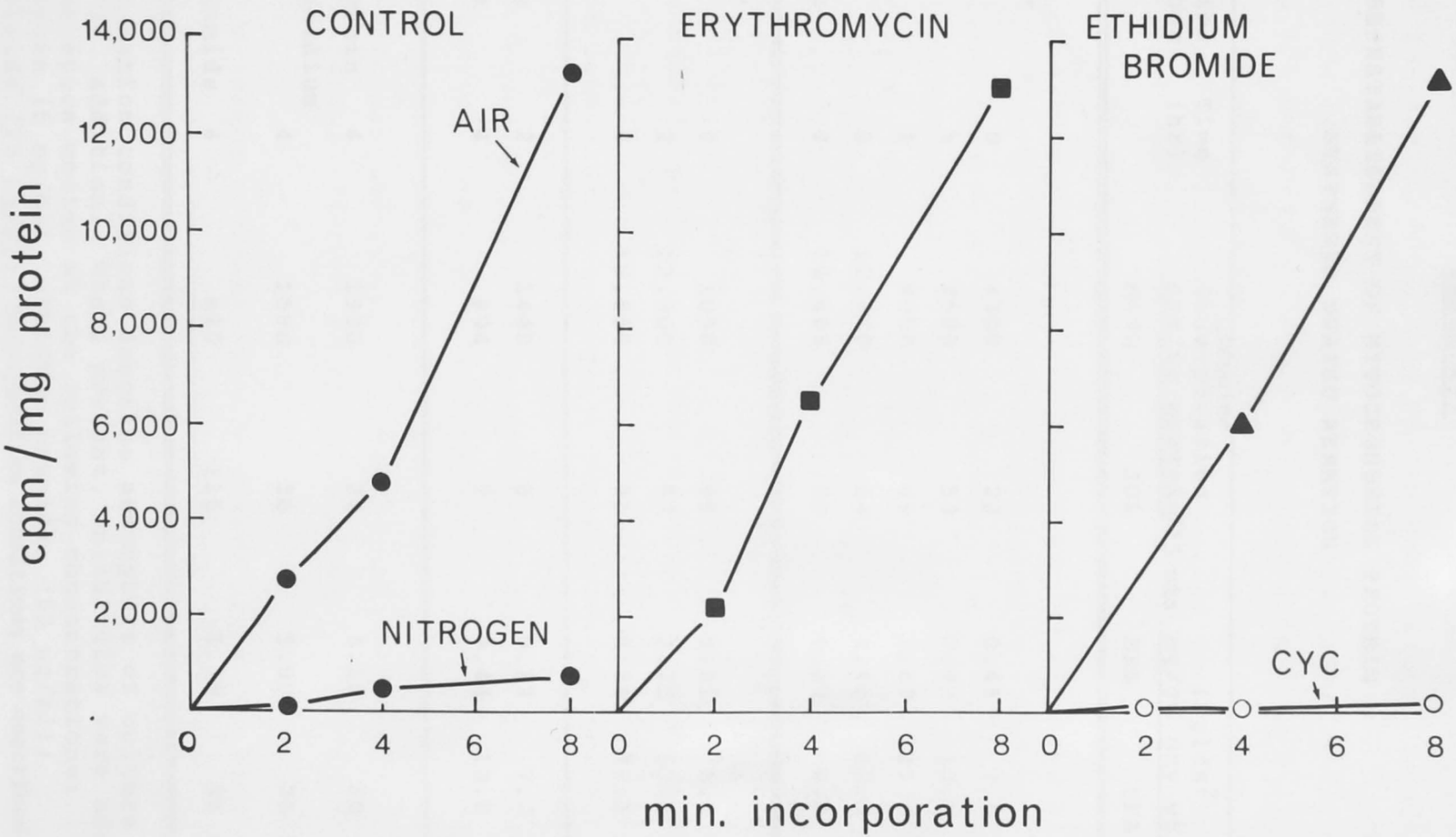

CONTROL
ERYTHROMYCIN
ETHIDIUM BROMIDE
AIR
NITROGEN
CYC
cpm / mg protein
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
2
4
6
8
min. incorporation

TABLE 4-7

RE-ESTABLISHMENT OF MITOCHONDRIAL PROTEIN SYNTHESIS DURING AERATION

Incubation conditions	Time (hr)	leucine Incorporation cpm/mg protein/15 min		Lipids[2] mg/gm dry wt.	
		MITO	SOL	ERG	UFA
Expt. 1.					
air	0	4300	23	0.45	7.5 (24)
air	½	9520	53	0.90	13.2 (36)
air	1	9910	49	1.47	21.6 (62)
air	2	12,780	84	2.46	27.3 (78)
nitrogen	2	11,445	50	0.47	4.8 (21)
Expt. 2.					
air	0	1050	95	0.21	5.3 (19)
air	2	12,900	57	2.72	53.8 (70)
air	4	18,850	91	4.24	71.2 (77)
nitrogen	2	1440	6	0.22	7.2 (17)
nitrogen	4	894	7	0.44	10.0 (20)
air + erythromycin	4	1930	34	5.10	80 (76)
air + ethidium bromide	4	1590	36	5.80	76 (77)
air + cycloheximide	4	420	115	1.39	36 (58)

1. Incubation conditions describe atmosphere of culture and antibiotic addition. Where present, antibiotics were added to the aeration medium at the following concentrations: erythromycin (5 mg/ml); ethidium bromide, (50 µg/ml); cycloheximide (25 µg/ml). Incorporation conditions are described in detail in Chapter II.

2. Lipids assayed were ergosterol (ERG) and unsaturated fatty acid (UFA). The values in parentheses are per cent UFA content (by weight) of the total fatty acids.

As indicated earlier, aeration of lipid-depleted cells resulted in large increases in the amounts of unsaturated fatty acid and ergosterol. In addition, there was considerable respiratory development so that after 4 hours aeration the respiration of the cells was more than half that of aerobically-grown, derepressed cells. Catalase activity was also assayed in these experiments as a marker for enzymic development in the cytoplasm; the activity of this enzyme increased 4-10 fold during 2 hours of aeration.

It is apparent (Table 4-7) that aeration resulted in the rapid re-establishment of the mitochondrial protein-synthesising system, without any obvious lag. The labelling of the (pro)mitochondrial fraction was inhibited about 90 per cent by erythromycin. The incorporation into the promitochondrial (zero time) fraction was found to vary considerably, but this was apparently related to the extent of lipid depletion of the anaerobically-grown cells; the more extensive the depletion the less labelling of promitochondrial protein. If the cells were incubated under nitrogen, rather than air, then over four experiments the extent of induction of protein synthetic activity was variable. In earlier experiments (e.g. experiment 1, Table 4-7) the induction under nitrogen was similar to that under air whereas in later experiments, where more care was taken during harvesting and resuspension of cells to exclude air, there was little induction (e.g. experiment 2, Table 4-7). The possible significance of these findings is discussed later.

The presence of cycloheximide during aeration, which caused a partial inhibition of induced lipid synthesis (see also chapter V), completely inhibited the development of mitochondrial protein synthetic activity (experiment 2). Apparently products of the cytoplasmic protein-synthesising system are required for this development. Erythromycin and ethidium bromide also inhibited this increase in activity of the mitochondrial system, however these results are more difficult to interpret. If the antibiotics were not washed

out of the cells before labelling, for example, then it is not possible to distinguish between a direct effect on protein synthesis activity and an effect on the development process. It should be noted that erythromycin, or ethidium bromide, have little effect on the development of the whole cell (cytoplasmic) protein-synthesising system, so the inhibition observed with these antibiotics must be attributed to an effect on the mitochondrial system.

9. RE-ESTABLISHMENT OF RNA-SYNTHESISING ACTIVITY DURING AERATION

The results presented earlier in this chapter indicated that RNA-synthesising activity, like protein synthesis, was lost during anaerobic growth in parallel with depletion of lipids from the cell. RNA synthesis was also rapidly re-established during aeration.

The rate at which this activity was regained, as measured by the rate of incorporation of ^{14}C-Uracil, was similar to that described for the regain of protein-synthesising activity.

DISCUSSION

1. EFFECTS OF LIPID DEPLETION DURING ANAEROBIC GROWTH OF *S. CEREVISIAE*

The early experiments of Andreasen and Stier (1953, 1954) defined unsaturated fatty acids and ergosterol as essential factors for the anaerobic growth of *S. cerevisiae*. However it is apparent from the results presented above that yeast cells will grow through several divisions anaerobically in the absence of added lipids: it is only when these essential lipids have been drastically decreased from levels found in the aerobic cell that growth slows and finally ceases. It is important to qualify what is meant by "growth" in these cultures. Even though cell division ceases after a relatively short time (10-12 hr) of anaerobic growth without lipids, cell mass and cell size continue to increase. A similar phenomenon ("unbalanced growth") is seen in bacteria when protein synthesis is inhibited by antibiotics (Hughes, Tanner and Stokes, 1970). Furthermore, even after the increase in cell mass has stopped (20-22 hrs), more than 95 per cent of the cells remain viable and capable of lag-free synthesis of lipid and protein. It appears that such cells are in a quiescent or dormant state after growth to lipid depletion.

If the cultures are allowed to age further cell viability starts to decline. This loss of viability may be important in considering the properties of depleted cells described by earlier workers (Hebb and Slebodnik, 1958; Morpurgo *et al.* 1964; Kovac *et al.* 1967; Criddle and Schatz, 1969). Criddle and Schatz (1969) have commented that their anaerobic lipid-depleted cultures were only 50 per cent viable, and Morpurgo and co-workers noted very unusual characteristics in such cultures.

It is difficult to define the exact cause of growth inhibition in lipid-poor anaerobic cultures. From the results presented above, it can be seen that anaerobic growth in

these cultures leads to the dilution of the cellular levels of unsaturated fatty acid and ergosterol. Practically all of these lipids present in the cells after anaerobic growth can be accounted for by the contribution from the lipids in the aerobic cells of the inoculum and the yeast extract in the medium. In addition, the dilution curve for the depletion of these lipids during anaerobic growth closely follows that predicted if the lipid content of the cells is halved at each division. The inference from this is that there is a little or no metabolic transformation of these lipids during anaerobic growth; this point is considered in more detail in chapter V.

Concomitant with the decrease in lipid levels is a decrease in the protein and RNA synthetic capacity of the cells. The ability of the cells to incorporate precursors into protein and RNA had decreased to about one third by the time cell division had ceased. It appears that in these cells protein and RNA syntheses, and cell division, are closely related to the synthesis or supply of the lipids unsaturated fatty acid and ergosterol. It is likely that the decreased protein and/or RNA synthesis, caused by lipid depletion, results in the inhibition of cell division.

2. EFFECT OF LIPID DEPLETION ON *IN VIVO* PROMITOCHONDRIAL PROTEIN SYNTHESIS

The results obtained using the *in vivo* labelling method of Schatz and Saltzgaber (1969a) confirms their finding that cells grown anaerobically with lipids contained an active (pro)mitochondrial protein-synthesising system. In lipid-depleted anaerobes, by contrast, activity due to promitochondrial protein synthesis was very low or absent. These *in vivo* results are thus consistent with the finding of Watson *et al.* (1970, 1971) and Davey *et al.* (1969), obtained with isolated promitochondria from lipid-depleted

and lipid-supplemented cells. However, the labelling studies of the lipid-depleted anaerobe suggest that the lack of activity of the promitochondrial system in this type of cell has to be carefully interpreted, as the cytoplasmic ribosomal system also appears to be inactive. The loss of protein synthesis is not a characteristic unique to the organelle, as it is in the petite mutant (Schatz & Saltzgaber, 1969a), and seems to be part of a general cellular quiescence.

3. EFFECT OF LIPID DEPLETION ON RIBOSOMES, RIBOSOMAL RNA, AND *IN VITRO* CYTOPLASMIC PROTEIN SYNTHESIS

The *in vitro* cytoplasmic protein synthesis studies confirm the results obtained *in vivo* in that the cytoplasmic protein-synthesising system of lipid-depleted anaerobic cells is inactive. The results obtained *in vivo* are thus not accounted for by lowered ATP levels or to very large increases in pool size in lipid-depleted cells. It can also be concluded that lipid depletion during anaerobic growth led to the inactivity of both the pH 5 enzyme/RNA fraction and the ribosome fraction. Furthermore, the inactivity of the *in vitro* system from lipid-depleted cells probably cannot be attributed to a lack of messenger RNA.

Recent work on the control of the biosynthesis of ribosomes in bacteria (Koch, 1970; Gray and Midgely, 1970) has shown that the rate of synthesis of ribosomal RNA is closely associated with the growth rate. Koch (1970) has concluded that "whatever mechanism controls the response of the cell to the nutritional state of the environment must involve control of the growth rate largely by adjusting the synthesis of ribosomal RNA". However these studies were carried out under conditions of balanced growth with growth rate controlled by media composition; in this chapter the comparison is between lipid-limited and lipid-supplemented yeast cultures which have been growing at different rates for a relatively short period. Certainly the lipid-limited

cultures have reached no such "balanced growth", as indicated by the growth curve and the radical changes in the lipid composition of the cells of this type of culture.

The measurements of RNA content of the two anaerobic cell types provide some insight into the growth characteristics of the cultures. It would be predicted from the bacterial studies discussed above that changes in the steady-state growth rate would lead to different RNA/protein ratios. But a comparison of the two types of anaerobic cultures have shown no differences in the specific RNA content of either the whole homogenates or of derived ribosome fractions. Further, there are no apparent differences in the polysome profiles, as noted above, nor are there marked differences in the species of RNA or their relative amounts when RNA is extracted from cytoplasmic fractions.

This analysis suggests that there has been no detectable adjustment of the amount of different RNA species in the lipid-depleted cultures to a slower rate of growth. It is interesting to note that these similarities in RNA content exist in spite of the loss of RNA synthesis, as measured by the *in vivo* incorporation of uracil, in lipid-depleted cells. Presumably, the increase in cell mass after division and RNA and protein synthesis have ceased is accompanied by a decreased rate of turnover of RNA and protein so that their relative amounts remain constant in the cells.

The reason(s) for the loss of protein synthetic activity during anaerobic growth without lipids is not clear. It is known that batch cultures of yeast, grown aerobically, lose their protein synthetic capacity as they approach stationary phase (Lucas, Schuurs, and Simpson, 1964; Bretthauer, Marcus, Chaloupka, Halvorson and Bock, 1963), and this has been related in *in vitro* systems primarily to a loss of the activity of the ribosome fraction. We have extended this finding to lipid-supplemented anaerobically-grown cells; on growth to stationary phase these cells lose their protein synthetic capacity. This loss appears to be

due to the disappearance of the polysome complement, which may be the consequence of a lack of initiation, and to the inactivity of the ribosomal fraction.

The loss of protein synthesis in lipid-depleted anaerobic cultures seems to have a different basis. There are two lines of evidence - the constancy of the amount and type of RNA, and the presence of normal polysomes - which indicate that this loss is not a consequence of the commencement of an early stationary phase due to lipid depletion. Therefore, if the integrity of the RNA species and the polysome structures have not been noticeably affected by lipid depletion at the time of harvesting then it is possible that the change in lipid composition during depletion has changed the lipid environment of the ribosomes, for example by changing the structure of the attached membranes, so as to make membrane-ribosome complexes non-functional or to decrease the proportion of membrane-bound ribosomes. This is supported by the decreased phospholipid content and changed phospholipid distribution of the ribosome fraction from lipid-depleted cells, relative to lipid-supplemented cells. Similar conclusions concerning the importance of attached membranes have been reached in studies on membrane-associated protein synthesis in bacterial systems (Schlessinger, 1963; Hindler, 1965, Moore and Umbreit, 1965) and non-bacterial systems(eg. Campbell, 1965, 1970; Fukuhara, 1967a). Moore and Umbreit (1965), for example, have shown that the protein synthetic activity of ribosome preparations from bacteria can be related to the phospholipid content of these fractions.

4. THE EFFECT OF LIPID DEPLETION ON THE SYNTHESIS OF MITOCHONDRIAL RNA

The results discussed earlier indicate that anaerobic growth without lipids leads to a marked decrease in the activity of the mitochondrial protein-synthesising system, whereas this system maintained its activity if lipids were

included in the anaerobic growth medium. This effect of lipids on mitochondrial protein synthesis can be correlated with the RNA content of the promitochondrial fraction from depleted and supplemented cultures - promitochondria from supplemented cells contain mitochondrial RNA species, while these appear to be absent from lipid-depleted promitochondria.

Forrester, Watson, and Linnane (1971a) have recently reported similar results, in that promitochondria from lipid-depleted cells, in contrast to the organelles from anaerobic lipid-supplemented cells and aerobic cells, appear to lack mitochondrial RNA. However, these workers found, on analysis (on sucrose density gradients) of RNA extracted from lipid-depleted promitochondria, a heterodisperse 6-14S band with a base composition similar to cytoplasmic RNA. The gel traces of RNA species that we have obtained from promitochondria of similarly-cultured cells showed only cytoplasmic RNA species. This may indicate that the heterodisperse peak obtained by Forrester *et al.* (1971a) represents degraded cytoplasmic RNA, and that their failure to observe any mitochondrial RNA from lipid depleted cells is due to degradation. The extension of these findings into a hypothesis by these workers suggesting regulation of ribosome formation by membrane lipid should therefore be viewed with caution.

Even so, the absence of mitochondrial RNA provides an explanation of the lack of mitochondrial protein synthesis in depleted cells. This is to be contrasted with the effect of lipid depletion on the cytoplasmic protein-synthesising system, where there appears to be no degradation of the ribosomes or RNA species. The above results may suggest that there is a differential turnover of mitochondrial and cytoplasmic RNA species, at least in depleted cells. Supporting this conclusion, Attardi and co-workers (Attardi *et al.* 1971) have found that in Hela cells mitochondrial species of RNA are most rapidly labelled. The situation may be similar to that of the DNA species of rat tissue, where mitochondrial DNA appears to have a much more rapid turnover

than nuclear DNA (Gross, Getz, and Rabinowitz, 1969). The analogy is strengthened by the fact that mitochondrial RNA appears to be coded for by mitochondrial DNA and synthesised in the mitochondria by a mitochondrial polymerase (see Küntzel, 1971, for review, and discussion in Chapter I).

5. THE RE-ESTABLISHMENT OF PROTEIN AND RNA SYNTHETIC ACTIVITY DURING AERATION

From the above discussion it is evident that wild-type cells grown anaerobically without lipids can be harvested during a period in the growth cycle where more than 95 per cent of the cells are viable, although lipid-depleted, and where there is little mitochondrial or cytoplasmic protein-synthesising activity. Such cells also have little capacity to synthesise RNA, though this does not seem to affect the RNA content, with the exception of mitochondrial RNA.

On aeration of these depleted cells the two protein-synthesising systems and whole cell RNA-synthetic activity are rapidly reformed, and there is a rapid lag-free synthesis of lipid (unsaturated fatty acids and ergosterol). These observations provide direct evidence in support of those presented by Vary *et al.* (1970), where it was found that depleted cells were capable of induced enzyme synthesis, the kinetics of which were similar to those found when lipid-supplemented anaerobic cells were aerated. The rapid re-establishment of protein and RNA synthesis also indicates that lipid-depleted cells, harvested as described, are in a quiescent state and that their inactivity is not a consequence of self-degradation.

This aeration system, in which the emergence of protein and RNA synthetic activities can be readily followed, provides an experimental system in which the factors responsible for the reactivation can be studied. In trying to define the events that occur on aeration we have examined two aspects in particular; (a) the requirement for products of protein

synthesis, and (b) the requirement for lipid synthesis.

Inhibitors of mitochondrial protein synthesis, such as erythromycin, or mitochondrial DNA transcription, such as ethidium bromide, had little effect on the development of the *cytoplasmic* protein-synthesising system. Hence it appears that products of the mitochondrial systems are not required for this reactivation, at least under these conditions. On the other hand the presence of these antiobiotics during aeration does inhibit mitochondrial protein synthesis, and this would suggest that products of the mitochondrial system are in fact required for the complete formation and development of its own activity. But, as discussed earlier, it is not possible at the moment to distinguish between a direct effect of these antibiotics on the activity, as opposed to the development, of the mitochondrial protein-synthesising system because of the difficulty of washing out the inhibitors prior to measurement of activity. In fact, Davey, Yu, and Linnane (1969) have concluded that the activity of the mitochondrial protein-synthesising system is not required for its formation, as the system is still active after growth in the presence of chloramphenicol.

Extensive hybridization data (reviewed by Küntzel, 1971), and the effect of ethidium bromide which inhibits mitochondrial DNA replication and transcription (Zylber Vesco and Penman, 1969; Goldring *et al.* 1970), has shown that mitochondrial ribosomal RNA is a product of mitochondrial DNA transcription (see Chapter I), and appears to be synthesised in the organelle. However it appears that the mitochondrial ribosomal proteins of *Neurospora* and yeast are synthesised on cytoplasmic ribosomes and then, presumably, transported into the mitochondrion (Davey, Yu, and Linnane, 1969; Küntzel, 1969; Neupert, Sebald, Schwab, Massinger, and Bücher, 1969). The results presented above, where cycloheximide inhibited the formation of the mitochondrial protein-synthesising system, are consistent

with these observations although proteins other than ribosomal proteins may also be involved. Cycloheximide also inhibits lipid synthesis during aeration by about 60 per cent (see also Chapter V), so it is possible that this inhibition prevents the development of the mitochondrial protein-synthesising system. This is unlikely, however, as it is possible in some instances to induce the formation of the mitochondrial system without concomitant lipid synthesis, as described below.

The question of the precise stimulus for the induction is an interesting one, and there are several possibilities. The experiments described involve taking cells that have been growing anaerobically, chilling them prior to harvesting, and after harvesting, resuspension in fresh medium at 29°: the cells are then aerated. It is possible that the induction stimulus is due to manipulation (e.g. chilling) or step-up culture conditions. However these possibilities can be ruled out as the adaption process can be effectively prevented (in most experiments) by incubation under nitrogen rather than air.

In the earlier experiments there was considerable induction of the mitochondrial (and cytoplasmic) protein-synthesising systems under nitrogen, though in the absence of lipid synthesis. It is possible that a small amount of oxygen was introduced in these experiments (particularly during harvesting and washing, when the cells were kept cold, though not rigorously anaerobic) and that this was enough to induce the formation of the protein-synthesising systems. This explanation implies that the processes responsible for the induction of protein synthesis and the induction of lipid synthesis have different thresholds for oxygen . In later experiments, where greater care was taken to exclude oxygen during the processing of cells, the extent of induction of the mitochondrial and cytoplasmic protein-synthesising system was very much less. It is hoped to test this hypothesis in the near future by supplying controlled amounts of oxygen to the adaption system.

Earlier it was suggested that lipid depletion during anaerobic growth caused the loss of mitochondrial RNA. The possibility raised by this observation is that the lack of mitochondrial RNA is at least part of the reason for the inactivity of the mitochondrial protein-synthesising system in these cells. This possibility is strengthened by the fact that we have found that mitochondrial RNA can be detected after a short period of aeration: similar findings have been made by Forrester, Watson, and Linnane (1971b). These workers (Forrester *et al.* 1971a) have proposed that mitochondrial ribosomal RNA synthesis and ultimately mitochondrial ribosome formation is directly dependent on the lipid composition of the membrane. The inference from this hypothesis is that the new mitochondrial RNA synthesis that is seen on aeration, and which is supposedly responsible for the re-emergence of mitochondrial protein synthesis, is a consequence of the new induced lipid synthesis. The results discussed above provide an interesting way of testing this hypothesis. If the adaption system can be controlled so that enough oxygen is admitted to induce mitochondrial protein synthesis, but <u>not</u> lipid synthesis, then the hypothesis must be discarded: preliminary results suggest that this is the case. The relationship between lipid synthesis and protein synthesis is further developed in the following chapters.

CHAPTER V

STUDIES ON THE CO-ORDINATION OF SYNTHESIS OF ENZYMES AND LIPIDS DURING RESPIRATORY ADAPTION.

I. THE EFFECTS OF CHLORAMPHENICOL AND CYCLOHEXIMIDE

INTRODUCTION

1. THE ANAEROBIC/AEROBIC TRANSITION

The important advantages of this experimental system have already been considered. Essentially, when anaerobically grown cells are exposed to oxygen they progressively acquire the ability to respire. This adaption involves the synthesis of respiratory enzymes and cytochromes, as well as lipid synthesis under certain conditions, and the integration of these components to form a functional (respiring) organelle. In the previous chapter the properties of anaerobically-grown yeast cells and the promitochondria isolated from these were described. The present chapter extends these studies to an examination of the synthesis of lipids and proteins that occurs on aeration of anaerobically-grown cells.

2. THE EFFECTS OF LIPID SYNTHESIS ON RESPIRATORY ADAPTION

Several groups of investigators have previously attempted to relate changes in the lipid content of whole yeast cells to changes that occur in the mitochondrion. Klein (1955) suggested a relationship between lipid synthesis and the synthesis of respiratory enzymes, although the only proof offered was that these were contemporaneous events when anaerobic yeast were aerated. Starr and Parks (1962) and Parks and Starr (1963) claimed that there was a close correlation between the suppression of sterol synthesis and the loss of respiratory capacity. On this basis, and

the then available information that anaerobically-grown yeast contained only small amounts of ergosterol and apparently no mitochondria, they proposed that ergosterol could quantitatively control the production of mitochondria. After further studies with inhibitors, Adams and Parks (1969) suggested that some protein essential for sterol synthesis was made by the mitochondrial protein-synthesising system, and that this component was oxygen-inducible. Kovac, Subik Russ and Kollar (1967), in contrast to the workers cited above, reported that concentrations of chloramphenicol which completely inhibited the development of respiration only partially inhibited lipid synthesis during aeration of anaerobic lipid-supplemented yeast.

At least two points need to be made concerning this earlier work. Firstly, in none of these studies were the lipids of the mitochondrial fraction examined. Secondly, the effects of the anaerobic growth conditions on the syntheses which occurred during subsequent aeration were not taken into account. Lack of consideration of the first point meant that no direct conclusions about mitochondrial development could be drawn, while ignorance of the effects of the anaerobic growth conditions on the physiology of the cells meant that important information was missed, as has been illustrated in the previous chapter.

The results presented in this thesis so far show that cells from two types of anaerobic culture, lipid-supplemented and non-supplemented, differ in their lipid composition, morphology, and biosynthetic capacity. Cells grown anaerobically without lipids have no active mitochondrial or cytoplasmic protein-synthesising systems, in contrast to the lipid-supplemented anaerobes. It might be expected on this basis that the behaviour of the anaerobic cells during aeration would be dependent on their previous growth history. Early studies of the induction process triggered by oxygen in yeast by Hebb and Slebodnik (1958) suggested that anaerobes grown on lipid-supplemented media adapted much faster than those grown in the absence of these supplements.

Morpurgo *et al.* (1964) found that lipid-depleted anaerobic cells were slower to develop respiration on aeration than were ergosterol-supplemented anaerobic cells. Although these results could not be confirmed (Vary *et al.*, 1970; and the results presented in this Chapter), the two types of culture have been found to differ significantly in other ways on aeration. This is well-illustrated by the differential effects of chloramphenicol on the induced synthesis of soluble mitochondrial enzymes (Vary *et al.* 1970).

3. THE USE OF CHLORAMPHENICOL AND CYCLOHEXIMIDE IN STUDYING THE CONTROL OF THE SYNTHESIS OF MITOCHONDRIAL COMPONENTS

In bacterial systems, chloramphenicol binds specifically to the 50S subunit of 70S-type ribosomes, and inhibits peptidyl transferase activity (Vazquez, Staehelin, Celma, Battaner, Fernandez - Munoz and Munro, 1969). Cycloheximide has been found to inhibit the elongation and initiation activities associated with the 80S-type ribosomes of fungal and animal cytoplasmic systems (Traub, 1969). In yeast, cytoplasmic ribosomes are 80S-type (Petermann, 1964; Chao and Klein, 1970), and *in vitro* cytoplasmic protein synthesis on these ribosomes is sensitive to cycloheximide and insensitive to chloramphenicol (Lamb, Clark-Walker, and Linnane, 1968). On the other hand, *in vitro* mitochondrial protein synthesis is sensitive to chloramphenicol and insensitive to cycloheximide (Lamb *et al.* 1968). These aspects have been discussed at length by Roodyn and Wilkie (1968) and Roodyn and Grivell (1969), for example. The inference that mitochondrial ribosomes are 70S-type has little basis. Studies so far on the size and structure of mitochondrial ribosomes show a wide range of sedimentation coefficients (Ashwell and Work, 1970; Borst and Grivell, 1971), so it is unlikely that the overall size of ribosomes is related to their response to specific inhibitors.

In vivo experiments with chloramphenicol and cycloheximide have led to rather unexpected results. For example, it has been shown that the synthesis of some mitochondrial enzymes can be inhibited by both chloramphenicol and cycloheximide. It is necessary to define 'mitochondrial protein synthesis' as either the synthesis of mitochondrial proteins (which may be carried out on cytoplasmic ribosomes), or the activity of the mitochondrial protein-synthesising system. The initial assumption that antibiotics such as chloramphenicol only block protein synthesis by the mitochondrial system *in vivo* has not been validated (for review see Ashwell and Work, 1970).

A number of recent studies on the biogenesis of mitochondria have yielded effects *in vivo* which are indicative of an extensive interaction between mitochondrial and cytoplasmic protein-synthesising systems (Hensen, Weber and Mahler, 1968a; Hensen, Perlman, Weber and Mahler, 1968b; Rouslin and Schatz, 1969; Vary *et al.* 1969; Vary *et al.* 1970). The results of these studies have suggested that under conditions where mitochondria are being formed, inhibition of one of these protein-synthesising systems will lead to some corresponding inhibition of the other system. Because of this coupling between the two systems it is difficult to distinguish their relative contributions to the synthesis of mitochondrial proteins *in vivo*.

In spite of the difficulty of interpreting the *in vivo* effects of inhibitors such as chloramphenicol and cycloheximide, they may still be used to considerable advantage. Providing side effects of these antibiotics can be eliminated or accounted for, they can be used to give information on the extent of coupling between the two protein-synthesising systems. Furthermore, these antibiotics offer an approach to the question of the integration of protein synthesis with the synthesis of non-proteins, such as lipids. It would be predicted that, in short term experiments and in the absence of non-specific effects, an

inhibition of the synthesis of non-proteins by these inhibitors would be the result of a coupling or inter-dependence between protein and non-protein syntheses, i.e. it would reflect a control mechanism coordinating these syntheses.

In the studies presented in this chapter we have examined the *in vivo* effects, at the intact cell and organelle level, of the inhibitors cycloheximide and chloramphenicol on the synthesis of mitochondrial components including both proteins and lipids. Careful consideration has been given to the importance of the anaerobic growth conditions, in particular the effects of lipid supplementation and the extent of growth. The rationale for choosing the lipid classes examined has been elaborated earlier.

RESULTS

1. CHARACTERISTICS OF THE AERATION SYSTEM

The conditions used in the following experiments, involving high-density cell suspensions in complex media, were chosen to provide an essentially non-growing population of cells and to minimize any step-down effects in the cultures. Non-growing aeration conditions provide several advantages. The contribution of the biosynthetic systems of the cell directed towards mitochondrial development is augmented as the cells are not concurrently generating large amounts of cell mass. This has been demonstrated by Fukuhara (1967a) in labelling studies on growing and non-growing cells. As well, precursor-product relationships between components of the anaerobic and aerobic cell can be more readily detected when dilution by growth is minimized. Finally, a non-growing system provides a better comparison with experiments where cycloheximide is used, as no growth occurs in the presence of this antibiotic.

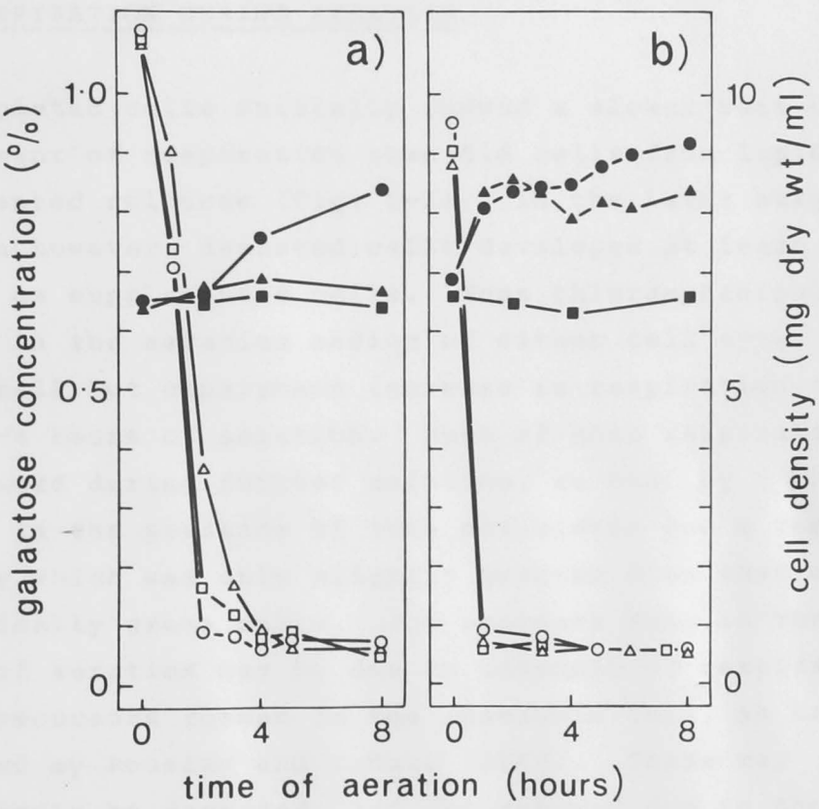
Galactose was used as energy source (and carbon source) for anaerobic and aerobic phases of these experiments because respiratory development during aeration was found to be both more rapid and more reproducible than when glucose was used as substrate. This is apparently a consequence of the less intensive catabolite repression of enzyme synthesis in yeast by galactose (Linnane, 1965; Polakis & Bartley, 1965).

The rate of galactose utilization and the changes in cell mass are shown in figure 5-1. The galactose utilization appeared to be more rapid during aeration of lipid-supplemented cells, although the galactose is depleted for both cell types under all the conditions used within three hours. The presence of the antibiotics had little effect on galactose utilization, except when lipid-depleted cells were aerated in the presence of chloramphenicol; disappearance of the sugar was then marginally slower. It can be seen that the desired non-growing conditions have been approximated in that the cells aerated without antibiotic completed less than one third of a division during the eight hour aeration period. There was no growth of either cell type in the presence of cycloheximide, or by depleted cells during aeration in the presence of chloramphenicol.

Although these aeration conditions resulted in an approximately linear synthesis of mitochondrial enzymes (Vary *et al.* 1970) it was found that the induced synthesis of lipids ceased after four hours. Nearly linear synthesis of lipids could be obtained if the galactose concentration was adjusted to one per cent after four hours aeration, and this was therefore adopted as standard procedure in the following experiments. This extra galactose had little effect on the development of respiration.

The viable cell count, determined by plating samples of the culture or by staining with buffered methylene blue (Gurr, 1965), showed that no significant cell death occurred during aeration with or without the antibiotics.

Figure 5-1



Galactose utilization and the increase in cell mass during aeration of anaerobically-grown cells. Cells were grown anaerobically on (a) non-supplemented or (b) lipid-supplemented 4% galactose medium and aerated in 1% galactose medium as described in Chapter II. Galactose concentration in the medium (open symbols), and cell mass (closed symbols), are indicated at intervals during aeration in the absence of antibiotic (O); in the presence of chloramphenicol, 4 mg/ml, (△); or in the presence of cycloheximide, 10 µg/ml, (□).

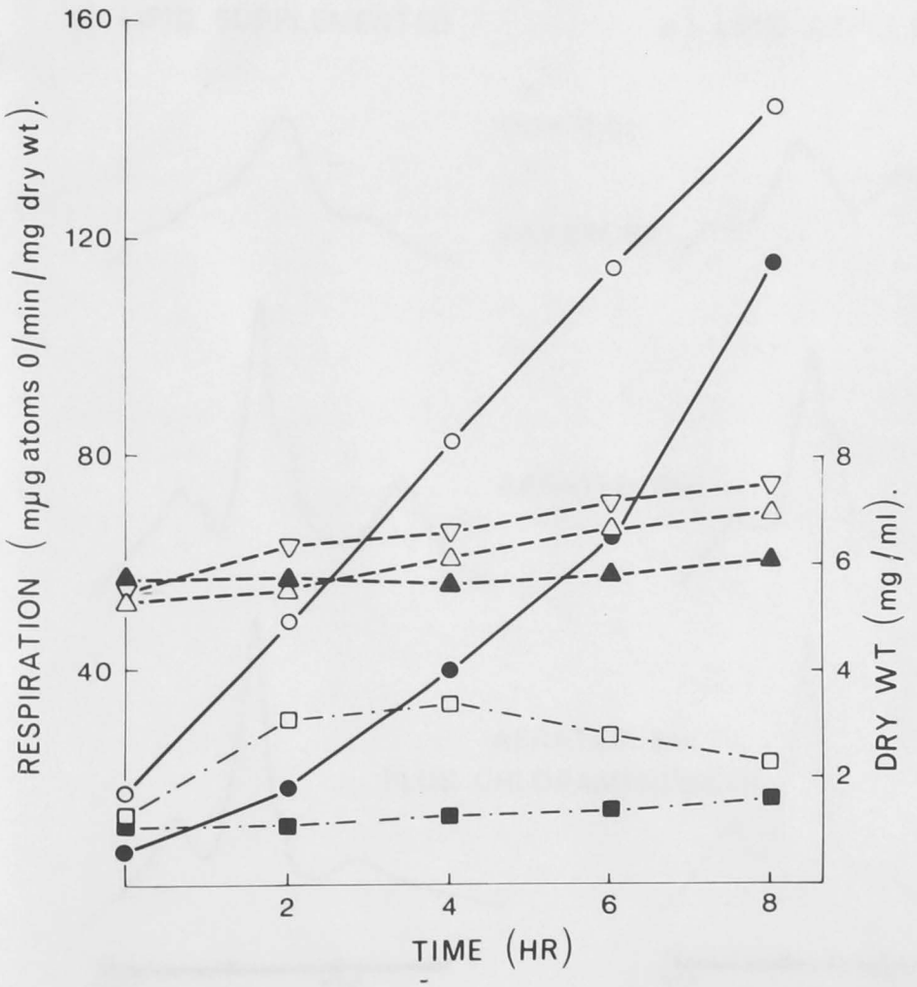
The only exception occurred during aeration of cells that had been grown anaerobically without lipids for extended periods (greater than 22 hours). In the following experiments cells have been grown anaerobically for 17-21 hours.

2. CYTOCHROME FORMATION AND THE DEVELOPMENT OF RESPIRATION DURING AERATION

Depleted cells initially showed a slower rate of development of respiration than did cells from lipid-supplemented cultures (Fig. 5-2). In the later stages of aeration however, depleted cells developed at least as rapidly as supplemented cells. When chloramphenicol was present in the aeration medium of either cell type, there was a small but consistent increase in respiration in the first 2-4 hours of aeration. Much of this respiration disappeared during further aeration, so that by 8 hr cells aerated in the presence of this antibiotic had a respiratory capacity which was only slightly greater than that of anaerobically grown cells. The increase seen in the early stages of aeration may be due to assembly of respiratory-chain precursors formed in the anaerobic cell, as has been suggested by Rouslin and Schatz (1969). These may subsequently be degraded, and not renewed due to the absence of mitochondrial protein synthesis. This suggested assembly of preformed respiratory elements would necessarily require synthesis of protein in the cytoplasm of the cell, since no significant increase in respiration occurred during aeration in the presence of cycloheximide (figure 5-2). The cell density figures shown emphasize the fact that there is little growth during the aeration period.

The spectral properties of anaerobically grown cells (Fig. 5-3) were similar to those described elsewhere (Wallace, Huang and Linnane, 1968; Ishidate, Kawaguchi, Tagawa and Hagihara, 1969, and papers referred to therein). During aeration of cells grown with lipid supplements there

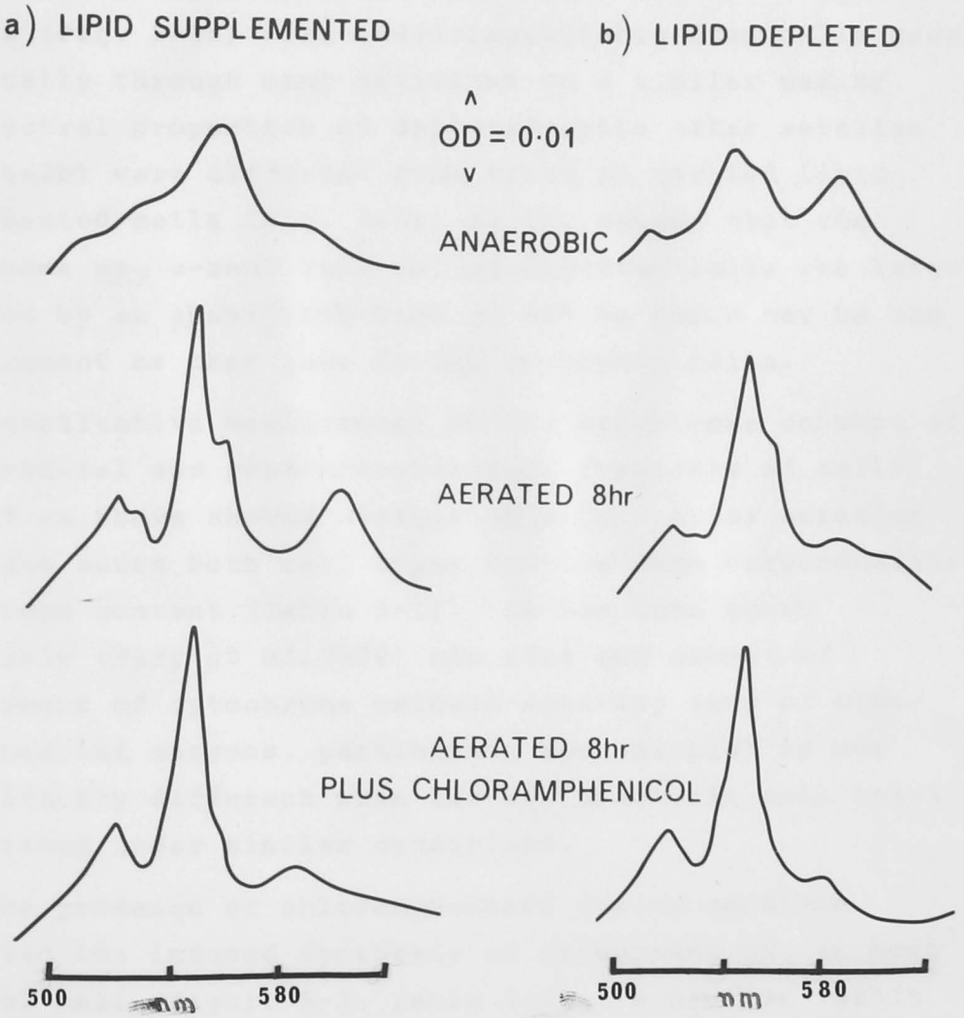
Figure 5-2



Oxygen-induced development of respiration in lipid-depleted and lipid-supplemented anaerobes. Respiration in supplemented cells aerated in the absence of antibiotic (O—O); in the presence of chloramphenicol (10 mM) (□—□); or in the presence of cycloheximide (20 μM) (■—■). Respiration in depleted cells aerated in the absence of antibiotic (●—●).

Cell density of supplemented cultures aerated in the absence of antibiotic (▽—▽); in the presence of chloramphenicol (10 mM) (△—△); or in the presence of cycloheximide (20 μM) (▲—▲).

Figure 5-3



Cytochrome content of cells grown anaerobically with or without lipid supplements, and after aeration in the presence or absence of chloramphenicol (10 mM).

was a rapid initial increase in the amount of cytochrome $\underline{c+c_1}$ (550 nm) in the cells, and a somewhat slower appearance of cytochrome $\underline{b}$ (562 nm) and cytochrome $\underline{aa_3}$ (603 nm). At the same time the absorption bands present in the anaerobic cells disappeared, or became obscured by the development of the aerobic cytochromes. After aeration for 8 hr the spectra (Fig. 5-3a) were indistinguishable from cells grown aerobically through many divisions on a similar medium. The spectral properties of depleted cells after aeration (Fig. 5-3b) were different from those of aerated lipid-supplemented cells (Fig. 5-3a) to the extent that the cytochrome $\underline{aa_3}$ α-band (603 nm) of depleted cells was largely obscured by an absorption band at 585 nm which may be the same pigment as that seen in the anaerobic cells.

Quantitative measurement of the cytochrome content of mitochondrial and post-mitochondrial fractions of cells aerated as above showed similar effects. After aeration for eight hours both cell types had the same mitochondrial cytochrome content (Table 5-1). As has been shown previously (Vary *et al.*1970) the rate and extent of development of cytochrome oxidase activity (and of other mitochondrial enzymes, particulate and soluble) is not significantly different when the two anaerobic cell types are aerated under similar conditions.

The presence of chloramphenicol during aeration prevented the induced synthesis of cytochrome $\underline{aa_3}$ in both types of cell (figure 5-3, Table 5-1). A similar result has been obtained by Vary *et al.*(1970) on an examination of cytochrome oxidase synthesis. The comparison is presented in Table 5-2, which shows the cytochrome $\underline{aa_3}$ content and the cytochrome oxidase activity of mitochondrial fractions prepared after aeration under the defined conditions. The relative inhibitions (Table 5-2), while following the same pattern, suggest that the increase in cytochrome content is more completely inhibited. This is particularly true in the case of cycloheximide treated fractions, and may suggest that the assay of enzyme activity is artificially high (or more sensitive).

TABLE 5-1

THE CYTOCHROME CONTENT OF FRACTIONS FROM AERATED CELLS : THE EFFECTS OF CHLORAMPHENICOL AND CYCLOHEXIMIDE

GROWTH CONDITIONS	Cytochromes					
	aa_3		b		$c + c_1$	
	MIT	SNT	MIT	SNT	MIT	SNT
1. grown anaerobically without lipids supplements						
aerated 8 hr.	0.31	<0.01	0.57	0.06	0.66	0.09
aerated 8 hr. + CAP	<0.01	<0.01	0.23	0.04	0.40	0.09
aerated 8 hr. + CYC	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
2. grown anaerobically with lipid supplements.						
aerated 8 hr.	0.28	<0.01	0.42	0.015	0.68	0.03
aerated 8 hr. + CAP	0.016	<0.01	0.09	0.01	0.38	0.04
aerated 8 hr. + CYC	<0.01	<0.01	0.06	0.01	<0.01	<0.01

Cells were grown anaerobically for 20 hr with or without lipid supplements, as indicated.

Chloramphenicol (CAP), 9mM, or cycloheximide (CYC), 20μM, were added at the start of aeration.

Cell fractions (mitochondria, MIT, and post-mitochondrial supernatant, SNT) were prepared by the spheroplast method. Values given are of a typical experiment; units are nmoles cytochrome/mg protein.

The absorption band at approximately 590 nm in lipid-supplemented anaerobes (Fig. 5-3,a) persisted during aeration in the presence of chloramphenicol, whereas it disappeared on aeration of control cultures. Since cytochrome $\underline{aa}_3$ formation is blocked by chloramphenicol, and dilution of precursor material by growth would be small, this band may represent the cytochrome a precursor suggested by earlier workers (Ephrussi and Slonimski, 1950; Ycas, 1956) or alternatively, may be residual cytochrome c peroxidase or cytochrome $\underline{a}_1$ of the anaerobic cell (Ishidate *et al.* 1969). The absorption band at 580-585 nm in lipid-depleted anaerobes persisted during aeration of both chloramphenicol-treated and control cultures (Fig. 5-3b). Because of the persistence during aeration of these absorption peaks present in the anaerobically-grown cells the cytochrome b levels are difficult to measure accurately. It is probable that the cytochrome b levels presented (Table 5-1) are high for this reason, particularly in the case of depleted cells. Nevertheless, it is possible to see that the effect of chloramphenicol on cytochrome b synthesis is similar to that exerted on cytochrome $\underline{aa}_3$ synthesis.

Of further interest was the fact that cytochrome c formation was little affected by chloramphenicol in either cell type during aeration; this is shown qualitatively by the whole-cell spectra (Figure 5-3). The synthesis of cytochrome c in lipid-supplemented cells in the presence of chloramphenicol thus resembles the induced formation of other soluble mitochondrial enzymes (Vary *et al.*1970). In lipid-depleted cells on the other hand, the synthesis of mitochondrial malate dehydrogenase and fumarase is inhibited completely by this antibiotic; cytochrome c formation, however, was largely unaffected (Fig. 5-3b; Table 5-1). Measurements in a typical experiment in which cytochrome c was extracted by the method of Sels *et al.* (1965) showed that the total amount of cytochrome c formed was 0.13 and 0.10 nmole/mg cell protein in control cells and chloramphenicol-treated cells respectively; 63% and 55%

TABLE 5-2

COMPARISON OF CYTOCHROME OXIDASE ACTIVITY WITH CYTOCHROME *aa*$_3$ CONTENT OF PARTICULATE FRACTIONS FROM AERATED CELLS

aeration conditions	cytochrome aa_3	cytochrome oxidase
1. aerated 4 hours	0.17 (100)	112 (100)
aerated 4 hours + CAP	0.02 (11)	34 (30)
aerated 4 hours + CYC	<0.01 (0)	39 (35)
2. aerated 8 hours	0.26 (100)	180 (100)
aerated 8 hours + CAP	0.02 (8)	(32) (18)
aerated 8 hours + CYC	<0.01 (0)	(31) (21)

Cells were grown anaerobically with lipid supplements, and mitochondrial fractions prepared after snail enzyme digestion Cytochrome content : nmoles/mg protein. Enzyme activity : nmoles cytochrome *c* oxidized/min/mg protein. Figures in parenthesis are values expressed relative to the appropriate control figure. Enzyme assays were carried out by Dr M. Lowdon. Chloramphenicol (CAP) and cycloheximide concentrations are as in Table 5-1.

* * * * * * *

respectively was recovered in the mitochondrial fraction. It appears, therefore, that the regulation of synthesis of this soluble cytochrome is different from that of either the TCA-cycle enzymes, or the particulate cytochromes (*aa*$_3$, *b*); the synthesis of the latter group is inhibited in both cell types (Figure 5-3), as is the case in batch-grown aerobic cultures (Huang *et al.* 1966).

Although the cytochrome spectra are not shown, cycloheximide prevented any significant qualitative change from those of the anaerobic cells. There was however a

decrease in the intensity of the bands. This inhibition of all cytochrome synthesis by cycloheximide is also shown in Table 5-1.

TABLE 5-3

ATPase ACTIVITY IN ANAEROBICALLY-GROWN CELLS, BEFORE AND AFTER AERATION. THE EFFECTS OF CHLORAMPHENICOL AND CYCLOHEXIMIDE.

Growth Conditions	Cell homogenate	MIT	SNT
1. Cells grown anaerobically with lipid supplements			
a. no aeration	22	70 (95)	12
b. aerated 8 hours	37	103 (98)	18
c. aerated 8 hours + CAP	40	108 (88)	17
d. aerated 8 hours + CYC	21	137 (95)	7
2. Cells grown anaerobically without lipid supplements			
a. no aeration	8	29 (90)	6
b. aerated 8 hours	38	104 (95)	6
c. aerated 8 hours + CAP	11	10 (20)	8
d. aerated 8 hours + CYC	5	9 (85)	1

Cells were grown anaerobically for 20 hr as described. Aeration conditions and antibiotic concentrations are given in Table 5-1. Cell fractions were prepared after cell rupture by the mechanical method. MIT is the 1000-15,000 g particulate fraction and SNT the supernatant of this particulate fraction. Values given are µgPi released/min/mg protein, and are typical values from at least three experiments. The figures in parentheses are the sensitivities to oligomycin, expressed as the percentage inhibition (see Chapter II).

3. THE SYNTHESIS OF ATPase(F_1) DURING AERATION

Somlo (1968) has reported that the mitochondrial ATPase of yeast is also oxygen-inducible, and the results presented in Table 5-3 agree in general terms with this observation. However it can also be seen that the presence of lipids during anaerobic growth is an important determinant of the basal ATPase activity, and thus of the relative induced synthesis of this enzyme. Hence, ATPase activity was lowest after anaerobic growth without lipid supplements, and aeration of this type of cell resulted in the greatest relative increase in ATPase activity. This effect of the anaerobic growth conditions on ATPase activity has also been reported by Criddle and Schatz (1969).

Table 5-3 also shows that after eight hours of aeration in the absence of antibiotics both cell types contained similar levels of ATPase activity. Chloramphenicol and cycloheximide had distinctive effects on ATPase synthesis. During aeration of lipid-supplemented cells cycloheximide prevented the increase in whole homogenate activity while chloramphenicol had little or no effect. Even so, cycloheximide did not prevent the increase in the specific activity of the mitochondrial fraction: this appeared to be due to a redistribution of the activity between mitochondrial and supernatant fractions. In lipid-depleted cells the effects of the antibiotics on the induced synthesis were quite different. Both chloramphenicol and cycloheximide inhibited the induced synthesis in homogenate and particulate fractions. In addition the ATPase formed in the presence of chloramphenicol in depleted cells was largely insensitive to oligomycin.

4. SYNTHESIS OF UBIQUINONE DURING AERATION

Aeration of both types of anaerobic cells resulted in the rapid synthesis of ubiquinone (Table 5-4) which in a period of 8 hr reached a level approaching that found in cells grown aerobically in batch culture (Chapter III).

TABLE 5-4

UBIQUINONE CONTENT OF CELLS AND MITOCHONDRIA AERATED IN THE PRESENCE OF ANTIBIOTICS

Conditions of anaerobic growth and aeration	Ubiquinone content:	
	Cells	Mitochondria
	(ug/mg protein)	
Lipid depleted		
Anaerobe	<0.01	<0.02
Aerated 4 hr	0.17	-
Aerated 4 hr + CAP[a]	0.08	-
Aerated 4 hr + CYC[a]	0.09	-
Aerated 8 hr	0.32	2.7 (84)
Aerated 8 hr + CAP	0.14	0.48 (36)
Aerated 8 hr + CYC	0.12	0.55 (44)
Lipid supplemented		
Anaerobe	<0.01	<0.02
Aerated 4 hr	0.22	-
Aerated 4 hr + CAP[a]	0.21	-
Aerated 4 hr + CYC[a]	0.12	-
Aerated 8 hr	0.34	2.7 (86)
Aerated 8 hr + CAP	0.31	1.4 (55)
Aerated 8 hr + CYC	0.16	0.58 (33)

Cells were grown anaerobically with or without lipid supplements, harvested,and aerated for the time indicated. Mitochondrial fractions were prepared from spheroplasts. Ubiquinone was extracted from mitochondria and from whole cells and estimated as described in the Methods. The figures in parentheses are the calculated percentages of total ubiquinone of the cell-free homogenate which is recovered in the mitochondria. This figure is a low estimate of the proportion of quinone located in the mitochondria. It is low because there is considerable entrapment and removal of mitochondria from the cell homogenates during the low-speed centrifugation to remove unbroken cells, nuclei and debris, (M.J. Lowdon, unpublished). [a]Chloramphenicol (CAP), 9mM, or cycloheximide (CYC), 20 µM, was added at the beginning of aeration.

Chloramphenicol barely affected the synthesis of ubiquinone during aeration of lipid - supplemented cells, and cycloheximide inhibited by about 50 per cent (Table 5-4). In the case of lipid-depleted anaerobes, both antibiotics inhibited synthesis during aeration by 60-70 per cent.

In both cell types the antibiotics brought about a proportionally greater reduction of the ubiquinone content of the mitochondrial fraction compared with whole cells (Table 5-4). This suggests that in addition to inhibiting the synthesis of ubiquinone the antibiotics either interfere with its incorporation into the organelle, or result in the formation of organelles with greater fragility so that during isolation they lose components that are normally tightly bound.

Since chloramphenicol and cycloheximide inhibit protein synthesis by mitochondria and by cytoplasmic ribosomes, respectively, it would appear that under non-growing conditions the synthesis of this mitochondrial quinone is not tightly linked to the synthesis of mitochondrial proteins, whether originating in the mitochondria or in the cytoplasm. Moreover, since neither antibiotic prevented the subsequent synthesis of ubiquinone when added at, or just prior to, the time of exposure of anaerobic cells to oxygen, it is likely that the enzymes of the ubiquinone biosynthetic pathway are constitutive in this organism, and are present in the anaerobic cell. Because of this, it might be expected that anaerobically-grown cells would accumulate polyprenyl phenol, as the operation of the pathway (as it has been defined in *E. coli*) to this point does not involve molecular oxygen. However, as shown in Chapter III, no polyprenyl phenol could be detected in anaerobically-grown cells.

5. SYNTHESIS OF LIPIDS DURING AERATION

(a) Synthesis of Phospholipids

Preliminary experiments indicated that there was considerable variation in the phospholipid content of cells grown anaerobically without lipid supplements. Figure 5-4 shows results from several experiments in which yeast cells (petite or wild-type) were grown to different stages of lipid depletion (as indicated by the level of unsaturated fatty acid). There appeared to be a linear relationship between the amount of unsaturated fatty acid and the amount of phospholipid in the cells. This suggests that the synthesis of phospholipid is limited by the availability of unsaturated fatty acid, and supports the postulate (Jollow *et al.* 1968) that there is a functionally significant interrelationship between phospholipid and unsaturated fatty acid levels. The variation in lipid levels after anaerobic growth probably reflects the difficulty of exactly reproducing growth conditions from experiment to experiment.

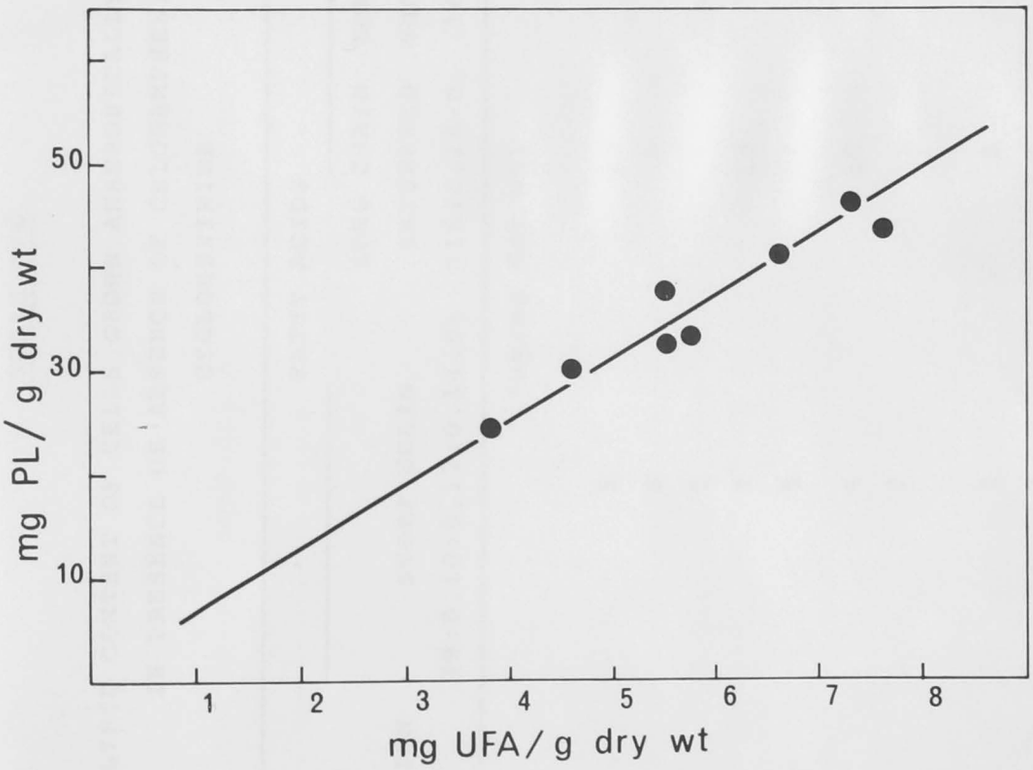
TABLE 5-5

EFFECT OF CHLORAMPHENICOL ON THE OXYGEN-INDUCED SYNTHESIS OF PHOSPHOLIPID IN DEPLETED CELLS

Growth Conditions	Cells (mg phospholipid/gm cells)	Mitochondria (µg phospholipid/mg protein)
Anaerobic	32	-
Aerated 8 hr.	50 (100)	155
Aerated 8 hr. + CAP[a]	39 (39)	62

Cells were grown anaerobically without lipid supplements for 20 hr, then aerated. Phospholipid was extracted from whole cells, or from mitochondrial fractions prepared after cell breakage by the mechanical method. Values in parentheses are percentages expressed relative to the increase in phospholipid observed in 8 hr in the absence of antibiotic.
a) Chloramphenicol (CAP), 9mM, was added at the start of aeration.

Figure 5-4



Relationship between phospholipid content and unsaturated fatty acid content after anaerobic growth without lipid supplements. Cells were grown to different stages of lipid depletion, as indicated in the text.

TABLE 5-6

LIPID CONTENT OF CELLS GROWN ANAEROBICALLY AND AERATED IN PRESENCE OR ABSENCE OF CHLORAMPHENICOL AND CYCLOHEXIMIDE

CONDITIONS OF GROWTH & AERATION	FATTY ACIDS				ERGOSTEROL
	SHORT CHAIN (8:0,10:0,12:0,14:0)	LONG CHAIN SATURATED (16:0,18:0)	LONG CHAIN UNSATURATED (16:1,18:1)	TOTAL	
	(µg/mg dry wt)				(µg/mg dry wt)
Lipid depleted					
Anaerobe	5	10	2	17	0.41
Aerated 4 hr	6	8	37	51	4.6
Aerated 4 hr + CAP[a]	5	7	15	27	1.1
Aerated 4 hr + CYC[a]	7	10	14	31	2.6
Aerated 8 hr	5	13	53	71	6.1
Aerated 8 hr + CAP	4	10	23	49	2.2
Aerated 8 hr + CYC	7	15	20	42	2.5
Lipid Supplemented					
Anaerobe	2	5	32	39	2.6
Aerated 4 hr	2	6	37	45	7.7
Aerated 4 hr + CAP	1	5	33	39	7.6
Aerated 4 hr + CYC	2	11	33	46	4.4

TABLE 5-6 (contd)

CONDITIONS OF GROWTH & AERATION	FATTY ACIDS				ERGOSTEROL
	SHORT CHAIN (8:0,10:0,12:0,14:0)	LONG CHAIN SATURATED (16:0,18:0)	LONG CHAIN UNSATURATED (16:1,18:1)	TOTAL	
	(µg/mg dry wt)				(µg/mg dry wt)
Aerated 8 hr	1	17	72	90	9.8
Aerated 8 hr + CAP	2	16	63	81	9.9
Aerated 8 hr + CYC	2	15	62	79	5.6
Aerobic	1	10	43	54	6.3

Cells were grown anaerobically and aerated in the presence of chloramphenicol or cycloheximide as described in Table 5-4. Unsaturated fatty acids and ergosterol were extracted and estimated as detailed in the Methods.

(a) Chloramphenicol (CAP), 9mM, or cycloheximide (CYC), 20 µM, was added at the beginning of aeration.

TABLE 5-7

EFFECT OF ANTIBIOTICS ON THE LIPID COMPOSITION OF MITOCHONDRIA FROM ANAEROBIC AND AERATED CELLS

CONDITIONS OF GROWTH & AERATION	FATTY ACIDS				ERGOSTEROL
	SHORT CHAIN (8:0,10:0,12:0,14:0)	LONG CHAIN SATURATED (16:0,18:0)	LONG CHAIN UNSATURATED (16:1,18:1)	TOTAL	
	(µg/mg dry wt)				(µg/mg dry wt)
Lipid depleted					
Anaerobe	40	113	22	175	<1
Aerated 8 hr	11	55	194	260	29
Aerated 8 hr + CAP[a]	13	48	129	190	32
Aerated 8 hr + CYC[a]	10	37	25	72	5
Lipid Supplemented					
Anaerobe	3	17	158	178	6
Aerated 8 hr	8	33	196	237	18
Aerobic	16	75	159	250	32

Cells were grown anaerobically and aerated in the presence of chloramphenicol or cycloheximide as described in Table 5-4. Mitochondrial fractions were obtained from cell homogenates prepared by the mechanical method.

(a) Chloramphenicol (CAP), 9mM, or cycloheximide (CYC), 20µM, was added at the beginning of aeration.

TABLE 5-8

EFFECT OF ANAEROBIC GROWTH AND AERATION CONDITIONS ON FATTY ACID COMPOSITION OF WHOLE CELLS

CONDITIONS OF GROWTH & AERATION	FATTY ACID[a] 8:0	10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1
	(weight % of total fatty acids)								
Lipid depleted									
Anaerobe	0.4	11.0	15.3	20.2	tr	37.2	5.2	6.8	3.9
Aerated 4 hr	tr[b]	3.6	6.9	6.7	4.8	12.2	34.4	6.2	24.9
Aerated 4 hr + CAP[c]	tr	7.6	11.1	10.1	6.8	21.0	25.4	6.6	11.5
Aerated 4 hr + CYC[c]	tr	3.9	7.0	7.7	4.1	21.0	27.4	9.5	19.2
Aerated 8 hr	tr	2.1	2.9	3.6	2.6	12.0	39.5	5.8	31.6
Aerated 8 hr + CAP	0.2	2.1	4.1	4.1	3.0	14.2	41.8	5.4	25.1
Aerated 8 hr + CYC	1.2	0.7	1.9	6.4	tr	33.4	22.1	14.7	19.6
Lipid supplemented									
Anaerobe	-[b]	0.2	0.4	3.4	1.2	14.5	13.6	1.1	65.8
Aerated 4 hr	-	0.3	0.3	2.0	1.3	9.9	24.6	3.8	58.0
Aerated 4 hr + CAP	-	0.3	0.5	2.4	1.4	9.7	22.4	2.2	61.2
Aerated 4 hr + CYC	-	0.2	0.4	2.7	1.0	19.2	14.9	3.8	58.0
Aerated 8 hr	-	0.2	0.5	1.0	0.9	6.5	25.8	1.5	63.6
Aerated 8 hr + CAP	-	0.2	0.5	1.0	1.1	7.3	26.0	2.1	60.8
Aerated 8 hr + CYC	-	0.2	0.6	2.9	1.0	18.3	13.3	4.2	59.6
Aerobic	-	0.2	0.3	1.1	tr	12.2	45.8	5.3	33.7

Experimental conditions are described in Table 5-4.

(a) Fatty acids are denoted by the convention:- number of carbon atoms: number of double bonds.

(b) tr indicates that the particular fatty acid represented less than 0.1% of the total fatty acid. - indicates that the amount of fatty acid was not measurable.

(c) Chloramphenicol (CAP), 9mM, or cycloheximide (CYC), 20 μM, were added at beginning of aeration.

TABLE 5-9

EFFECT OF ANAEROBIC GROWTH AND AERATION CONDITIONS ON FATTY ACID COMPOSITION OF THE MITOCHONDRIAL FRACTION

CONDITIONS OF GROWTH & AERATION	FATTY ACID[a] 8:0	10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1
Lipid depleted									
Anaerobe	0.6	1.4	7.2	13.8	0.4	54.7	5.7	9.7	6.5
Aerated 8 hr	0.1	0.4	1.3	2.4	0.4	18.1	43.5	3.0	30.8
Aerated 8 hr + CAP[b]	0.2	0.8	1.9	3.8	0.9	22.6	40.1	2.8	26.9
Aerated 8 hr + CYC[b]	0.3	1.5	2.7	9.4	0.4	43.0	26.6	8.1	8.0
Lipid supplemented									
Anaerobe	tr[c]	2.1	2.5	3.7	0.0	29.4	8.5	18.5	35.3
Aerated 8 hr	1.3	0.3	0.6	1.5	0.8	12.9	37.6	3.0	42.1
Aerobic	0.7	2.0	2.2	2.5	1.0	15.7	36.8	11.1	28.0

Experimental conditions are described in Tables 5-4 and 5-7.

(a) Fatty acids are denoted by the convention:- number of carbon atoms : number of double bonds.

(b) Chloramphenicol (CAP), 9 mM, or cycloheximide (CYC), 20 µM, were added at beginning of aeration.

(c) tr indicates that the particular fatty acid represented less than 0.1% of the total fatty acid.

When these anaerobically-grown lipid-depleted cells were aerated, the increase in phospholipid content was never greater than twofold (Table 5-5). Nevertheless, it was obvious that chloramphenicol was able to greatly inhibit this induced synthesis, and this was true both of whole cells and mitochondria (Table 5-5). This inhibition of lipid synthesis by chloramphenicol was even more apparent when the induced synthesis of unsaturated fatty acid and ergosterol was examined.

(b) Synthesis of Unsaturated Fatty Acids and Ergosterol

Cells grown anaerobically without lipid supplements contained small amounts of unsaturated fatty acids and sterol compared with aerated or aerobically grown cells (Table 5-6), confirming the results of Jollow *et al.* (1968). (Pro)mitochondria from anaerobic depleted and aerated cells showed similar substantial differences in lipid content (Table 5-7). When depleted cells were aerated the increase in unsaturated fatty acid content of the mitochondrial fraction occurred concurrently with a decline in amount of saturated fatty acids in this fraction. It therefore appears that desaturation and elongation of preexisting acids occur. However, the greater part of the increase in unsaturated fatty acid content of both cell and organelle, induced by aeration, represents a net increase in total fatty acids, indicating substantial *de novo* synthesis. In short term experiments (2 hr aeration) desaturation and elongation of existing fatty acids may be the major mechanisms (see Chapter IV).

Detailed analyses of the fatty acid composition of cells and of (pro)mitochondria from these cells are given in Tables 5-8 and 5-9.

Lipid-supplemented anaerobes after aeration contained considerably more unsaturated fatty acids and sterol than cells grown batch-wise in the presence of oxygen. However,

these excess levels of cellular lipid are not accounted for by abnormally large amounts of lipid in mitochondria prepared from these cells (Table 5-7), nor to any notable increase in the amount of mitochondrial protein in these cells; there was no significant difference in the proportion of cell protein in mitochondria of these two cell types. This excess lipid presumably represents storage lipid synthesised during aeration.

The effects of chloramphenicol and cycloheximide on lipid synthesis during aeration of depleted cells (Table 5-6) were in general terms similar to the effects seen on ubiquinone synthesis and phospholipid synthesis. Both antibiotics inhibited ergosterol and unsaturated fatty acid synthesis by about 60 per cent. Examination of the mitochondrial fractions from these aerated cells (Table 5-7) showed that the inhibition of unsaturated fatty acid synthesis by chloramphenicol was similar to that seen in whole cells. On the other hand, the effect of cycloheximide was greater on the lipids of the mitochondrial fraction than on the whole cells.

In lipid-supplemented anaerobes, the higher initial levels of lipid largely obscured any effects that the antibiotics might have had on lipid synthesis during subsequent aeration. There did, however, appear to be an effect of cycloheximide, but not chloramphenicol, on the oxygen-induced sterol synthesis in these cells.

6. SENSITIVITY OF INDUCIBLE ENZYME AND LIPID SYNTHESIS TO CHLORAMPHENICOL AND ERYTHROMYCIN

Results presented in the above sections showed that the synthesis of lipids (unsaturated fatty acids, phospholipids, ergosterol, ubiquinone) that was induced when lipid-depleted anaerobic cells were aerated was subject to inhibition by both chloramphenicol and cycloheximide, to the extent of about 60 per cent. Vary *et al.* (1969, 1970) have reported

that in the same aeration system the induced formation of mitochondrial enzymes is also inhibited by these antibiotics. In order to compare these inhibitions of lipid and enzyme synthesis, we have examined the extent of the induced synthesis as a function of antibiotic concentration. Because cycloheximide inhibited enzyme synthesis completely at all the concentrations tested (down to 1 µg/ml), only the results obtained from experiments with inhibitors of mitochondrial protein synthesis are presented. Both chloramphenicol and erythromycin have been used in the following experiments. These antibiotics have different sites of action as inhibitors of protein synthesis and have somewhat different effects on respiration and phosphorylation. For example, we have found that with α-ketoglutarate as substrate, the chloramphenicol isomers (D - and L-*threo*) inhibited phosphorylation severely while erythromycin had little effect. It was hoped that a quantitative comparison of the effects of these two antibiotics would help to eliminate non-specific side effects. In the following experiments cytochrome oxidase was assayed as being representative of the membrane bound cytochromes, succinate dehydrogenase as being typical of membrane - bound flavoproteins, and fumarase as representative of soluble, matrix - located enzymes (for discussion of mitochondrial enzyme localization see Ernster and Kuylenstierna, 1969). The unsaturated fatty acids, palmitoleic and oleic, and the sterol ergosterol were measured as being indicative of the lipid changes associated with mitochondrial development.

The extent of the induced synthesis of these enzymes and lipids during aeration for 4 hr as a function of antibiotic concentration present during the aeration phase is shown in figure 5-5,a and b. The induced formation of enzymes (Vary *et al.* 1970) and lipids (Chapter VI) is essentially linear during an 8 hr. aeration period. However, considerable induction has taken place after 4 hours aeration so this has been the aeration period selected to study the inhibitor effects. Clearly, the induced formation of

figure 5-5

Effects of inhibitor concentration on the oxygen-induced synthesis of lipids and mitochondrial enzymes :

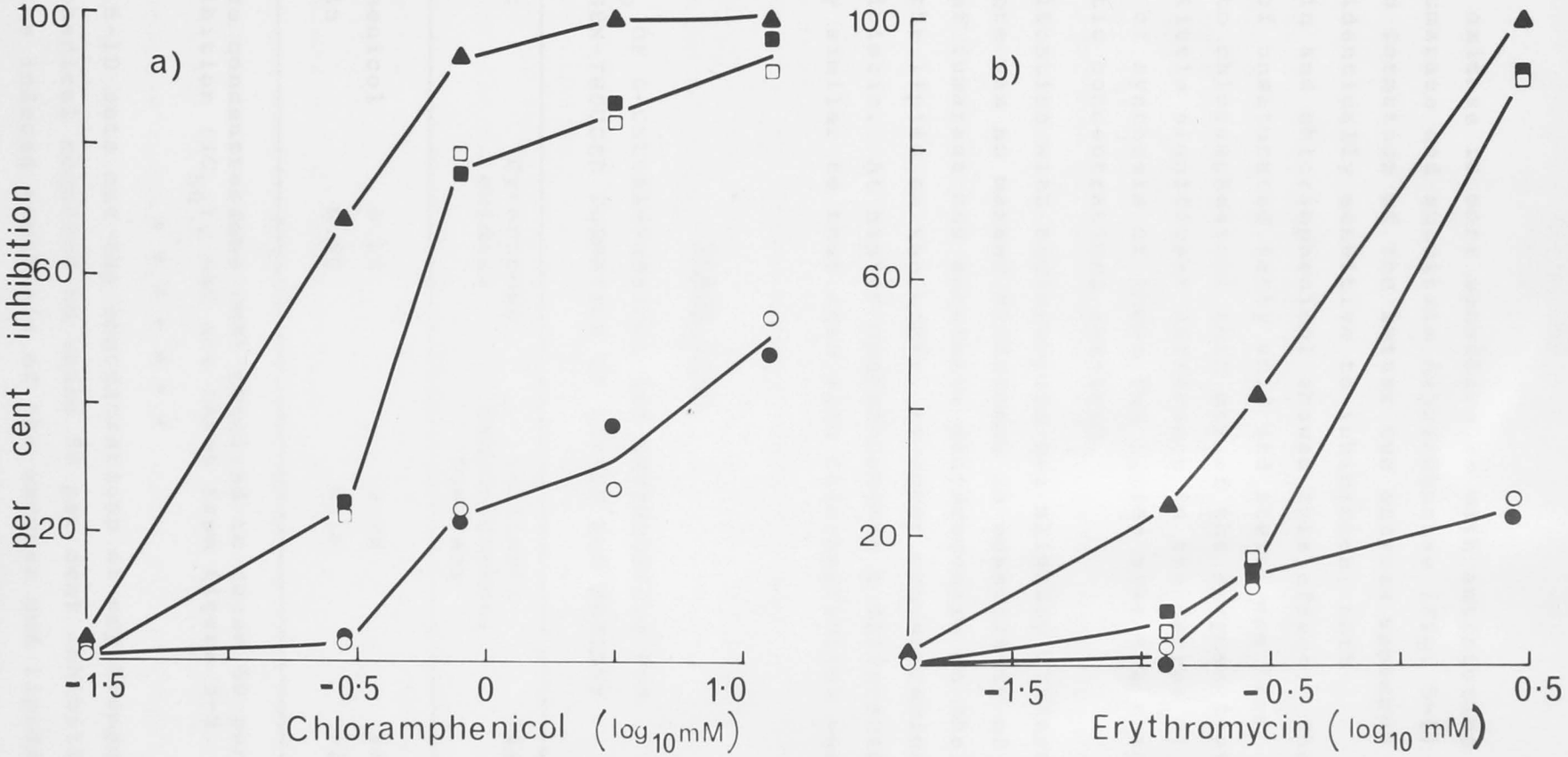
a) response to chloramphenicol;

b) response to erythromycin.

Cells were grown anaerobically without lipid supplements for 20 hr, then aerated for 4 hr in the presence of the antibiotic concentration indicated. Cells were then analysed for lipid content, or broken in the Frence press for the preparation of a <u>whole homogenate</u> which was used for the enzyme assays; these assays were carried out by Dr. M. Lowdon.

▲	cytochrome oxidase	(0.108)
■	fumarase	(0.159)
□	succinate dehydrogenase	(0.140)
●	unsaturated fatty acid	(40)
O	ergosterol	(4.7)

Values in parentheses are control levels for the two experiments. The units of the enzyme activities are μmoles oxidized / min / mg protein, or O.D.U. change / min / mg protein in the case of fumarase : the lipid levels are expressed as mg / gm dry wt cells.


a)
b)
100
60
20
per cent inhibition
-1·5
-0·5
0
1·0
0·5
Chloramphenicol (log10 mM)
Erythromycin (log10 mM)

cytochrome oxidase is more sensitive to both antibiotics than are fumarase and succinate dehydrogenase (Fig. 5-5). The induced formation of the latter two enzymes appeared to be almost identically sensitive to inhibition; both erythromycin and chloramphenicol showed this effect. The synthesis of unsaturated fatty acid and sterol was less sensitive to chloramphenicol than any of the enzymes tested. There was little significant difference in the degree of inhibition of synthesis of these two lipids over the range of antibiotic concentrations examined.

The situation with erythromycin was slightly different, in that there was no marked difference in sensitivity of the induction of fumarase and succinate dehydrogenase on the one hand, and the lipids on the other, at lower concentrations of the antibiotic. At higher concentrations a differential sensitivity similar to that seen with chloramphenicol was apparent.

TABLE 5-10

IC_{50} OF D-CHLORAMPHENICOL AND ERYTHROMYCIN FOR OXYGEN-INDUCED FORMATION OF LIPIDS AND ENZYMES

Antibiotic	Cytochrome oxidase	Succinate dehydrogenase, fumarase	Lipids
D-Chloramphenicol	0.16	0.68	11.5
Erythromycin	0.40	0.78	>2.7

Values are concentrations (mM) required to cause 50 per cent inhibition (IC_{50}), and are taken from figure 5-5.

* * * * * * *

Table 5-10 sets out the concentrations of erythromycin or chloramphenicol required to cause 50 per cent inhibition (IC_{50}) of the induced synthesis of the enzymes and lipids. The different levels of sensitivity to the antibiotics described above are evident. Furthermore the IC_{50} for the

two antibiotics in each group were similar on a molar basis : the solubility limit of erythromycin base (approx. 2.8mM) prevented a more accurate assessment of the IC_{50} of this antibiotic on lipid synthesis.

The induced synthesis of mitochondrial enzymes and lipids examined above show a hierarchy of responses to inhibitors of mitochondrial protein synthesis. The meaning of these results is not immediately obvious. It is possible, for example, that the results indicate that there is an inhibitory response of mitochondrial protein synthesis (as shown by cytochrome oxidase), and because lipid synthesis and the synthesis of other enzymes are incompletely coupled to mitochondrial protein synthesis, inhibition of these is also observed, but to a lesser degree. Interpretations such as this are complicated by questions concerning the specificity of the antibiotics used.

The possibility that non-specific effects of chloramphenicol, for example, resulting from inhibition of respiration or phosphorylation (Beattie 1968; Freeman and Haldar, 1968), could account for the inhibition observed in the wild type was examined by using the stereoisomer L(+) - *threo*-chloramphenicol. This isomer is inactive as an inhibitor of bacterial protein synthesis (Brock, 1961) but inhibits mitochondrial respiration and uncouples phosphorylation to a similar extent to the D(-) - *threo* isomer (Beattie, 1968; Freeman and Haldar, 1968). Table 5-11 shows the results of an experiment in which the effects of the chloramphenicol isomers on the development of respiration and oxygen-induced lipid synthesis are compared. The inhibitory effects of the D-isomer are not observed when the L-isomer is used. The L-isomer is also without effect on induced enzyme synthesis in these cells (Vary *et al.* 1970). The inhibitions by D(-) - *threo*-chloramphenicol, the active isomer, can therefore be attributed to an inhibition of protein synthesis in the cell, rather than to some other less direct effect.

TABLE 5-11

EFFECT OF CHLORAMPHENICOL ISOMERS ON DEVELOPMENT OF RESPIRATION AND SYNTHESIS OF LIPIDS INDUCED BY AERATION

Aeration Conditions	Unsaturated fatty acids	Phospholipid	Respiration (ngat. Oxygen /min/mg prot.)
	µg/mg dry wt. cells		
(Anaerobe	5.5	33	7
Aerated 8 hr.	63	63	107
Aerated 8 hr. + D-CAP (10mM)	30	43	10
Aerated 8 hr. + L-CAP (10mM)	65	60	118

Cells were grown anaerobically without lipid supplementation for 19 hr, then harvested and aerated in the presence of the D- or L-isomer of chloramphenicol (CAP).

TABLE 5-12

EFFECT OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON THE DEVELOPMENT OF MITOCHONDRIAL STRUCTURE DURING AERATION

Cell type	Mitochondria/cell section		Total
	Without cristae	With cristae	
Anaerobic	3*	0.5	3.5
Aerated	1.5	5.7	7.2
Aerated + chloramphenicol	1.5	3.4	4.9
Aerated + cycloheximide	4.9	0.2	5.1

Cells were grown anaerobically with lipid supplementation, harvested and aerated with or without chloramphenicol or cycloheximide as described in Table 5-9. After aerating for 8 hr samples were removed, fixed, and stained for examination in the electron microscope. 15-30 sections of each cell type (containing a well-defined nucleus) were scored for the presence of mitochondrial structures with and without discernible cristae.

* poorly-defined

7. THE EFFECT OF ANTIBIOTICS ON THE DEVELOPMENT OF MITOCHONDRIAL MORPHOLOGY

Cells grown anaerobically with or without lipids were aerated in the presence or absence of chloramphenicol or cycloheximide. The cells were then fixed with permanganate, post-fixed and stained with osmium tetroxide, and the ultrastructure examined by electron microscopy. Preliminary examination of the micrographs obtained showed that during aeration of lipid-depleted cells both antibiotics inhibited the formation of mitochondria and the development of cristae; cycloheximide inhibited cristae development practically completely. However, since these antibiotics also strongly inhibit lipid synthesis in this type of cell, and because permanganate fixation depends largely on the lipid composition of the membranes (Chapter I), it is difficult to interpret the effects on morphology using this particular fixation procedure.

It is probably valid only to compare the morphology of permanganate-fixed cells when the lipid composition of the cell types is similar. This condition is approximated in the system involving aeration of lipid-supplemented cells. Table 5-12 shows the effects of the antibiotics on mitochondrial formation during aeration of this type of cell. The most obvious effects are seen on cristae development. Again, cycloheximide appears completely to prevent cristae formation. As antibiotic effects on lipid synthesis can be largely discounted in lipid-supplemented cells, it would seem that the synthesis of some protein component(s) are required for the development of cristae.

8. CONSERVATION OF PROMITOCHONDRIAL MEMBRANES DURING AERATION

The flexibility in the requirement for unsaturated fatty acids (Light, Lennarz, and Bloch, 1962) and sterol (Proudlock *et al.* 1968) in *S. cerevisiae*, discussed in Chapter I, makes this organism a useful one in which to study structure/function relationships of these lipids.

The dilution rate of these lipids during anaerobic growth of *S. cerevisiae* (Chapter IV) suggested that there was a very limited turnover of these lipids. A similar inference can be obtained from another set of data. The relative proportions of oleic (18:1) and palmitoleic (16:1) acids in anaerobic, supplemented cells was found to reflect the relative amounts of these unsaturated fatty acids in the Tween 80 supplying these essential growth factors. This was also noted by Jollow *et al.* (1968), and suggests that there is limited metabolic transformation of unsaturated fatty acids after incorporation into the anaerobic cell. Thus the ratio of oleic to palmitoleic in cells grown anaerobically with this supplement is about 5 to 1 (Table 5-13), compared with 10.1 to 1 in the Tween 80 used, and less than 0.8 to 1 in aerobic cells which synthesise their own fatty acids. Even after aeration for 8 hr. the lipid-supplemented cells have a higher proportion of oleic, whereas lipid-depleted cells after aeration have a fatty acid composition the same as aerobically-grown cells. These different ratios of oleic to palmitoleic acid were also evident in the (pro)mitochondria from these cells (Table 5-13). Interestingly, in lipid-depleted anaerobically-grown cells oleic acid is concentrated in the promitochondrial fraction : this may reflect a selective requirement for this fatty acid in mitochondrial membranes. A similar variability could be demonstrated with the sterols by supplying, for example, the non-yeast sterol cholesterol as the sterol growth factor for anaerobic growth (cf. Proudlock *et al.* 1968).

The extent to which unsaturated fatty acid and sterol components of promitochondrial membranes were conserved during respiratory development was therefore measured as follows. Cells were grown anaerobically with linoleic acid (18:2) and cholesterol as lipid growth factors. Both of these lipids are absent from aerobic cells, even though together they support anaerobic growth, and thus can be readily distinguished from lipids that are synthesised on aeration. After anaerobic growth on these lipids, more than 90 per cent of the unsaturated fatty acid content was

accounted for as linoleic acid, and more than 75 per cent of the sterol as cholesterol. The anaerobically-grown cells were harvested, washed extensively, and aerated for 8 hr. At intervals, cell samples were removed and mitochondrial fractions prepared. Sterol and fatty acid analyses of cells and mitochondria gave the results summarized in Figures 5-6 and 5-7.

TABLE 5-13

UNSATURATED FATTY ACID COMPOSITION OF ANAEROBIC AND AERATED CELLS AND OF MITOCHONDRIA DERIVED THEREFROM

Cell Type	Percentage of Total Fatty Acid Present as 16:1	18:1	Ratio 18:1/16:1
ANAEROBIC			
Lipid Depleted:			
Cells	6	4	0.67
Promitochondria	5	12	2.40
Lipid Supplemented:			
Cells	13	65	5.00
Promitochondria	9	35	3.90
AERATED			
Lipid Depleted:			
Cells	46	34	0.74
Mitochondria	43	31	0.72
Lipid Supplemented:			
Cells	23	61	2.65
Mitochondria	40	42	1.05
AEROBIC, BATCH GROWN			
Cells	46	34	0.74
Mitochondria	45	32	0.71
TWEEN 80	9	91	10.10

Cells were grown anaerobically or aerobically, with or without lipid supplements, as described. Fatty acids tabulated are oleic acid (18:1) and palmitoleic (16:1), as measured after GLC analysis.

figure 5-6

Unsaturated fatty acid content of cells, and mitochondria from these cells, grown anaerobically on linoleic acid and cholesterol and aerated in the absence of these lipids.

Cells were grown anaerobically with linoleic acid (600 µg/ml) and cholesterol (20 µg/ml) replacing the usual lipid supplements (Tween 80 and ergosterol). After harvesting and extensive washing, the cells were aerated in media containing no added lipid. Samples were removed at the times indicated and mitochondrial fractions prepared. Fatty acid and sterol analysis was carried out on whole cells and on the isolated mitochondria. Cholesterol and ergosterol accounted for more than 90% of the total sterol in each case, and linoleic, oleic and palmitoleic acids for more than 98% of the unsaturated fatty acids extracted. Development of respiration during aeration was not significantly different from that shown for lipid supplemented cells in figure 5-2.

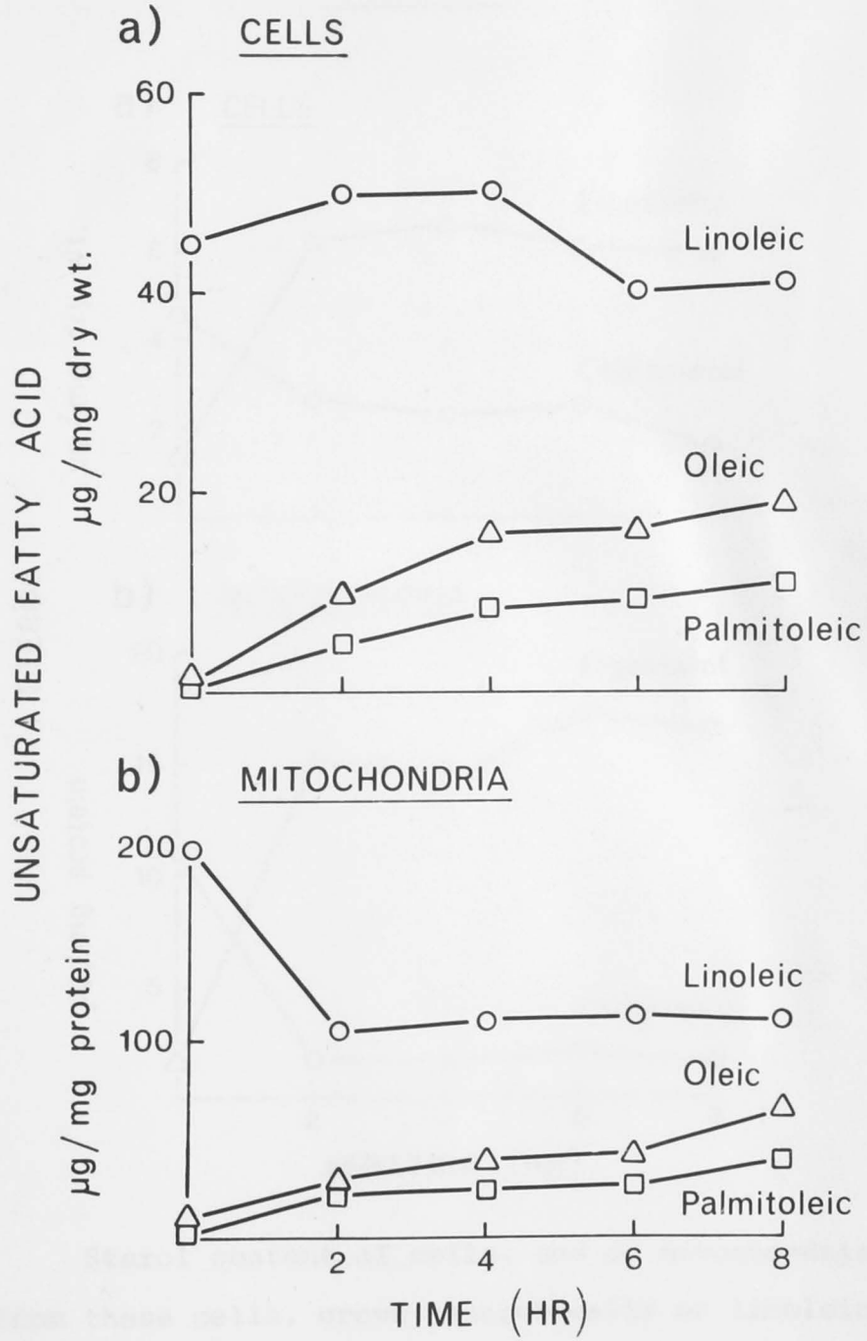
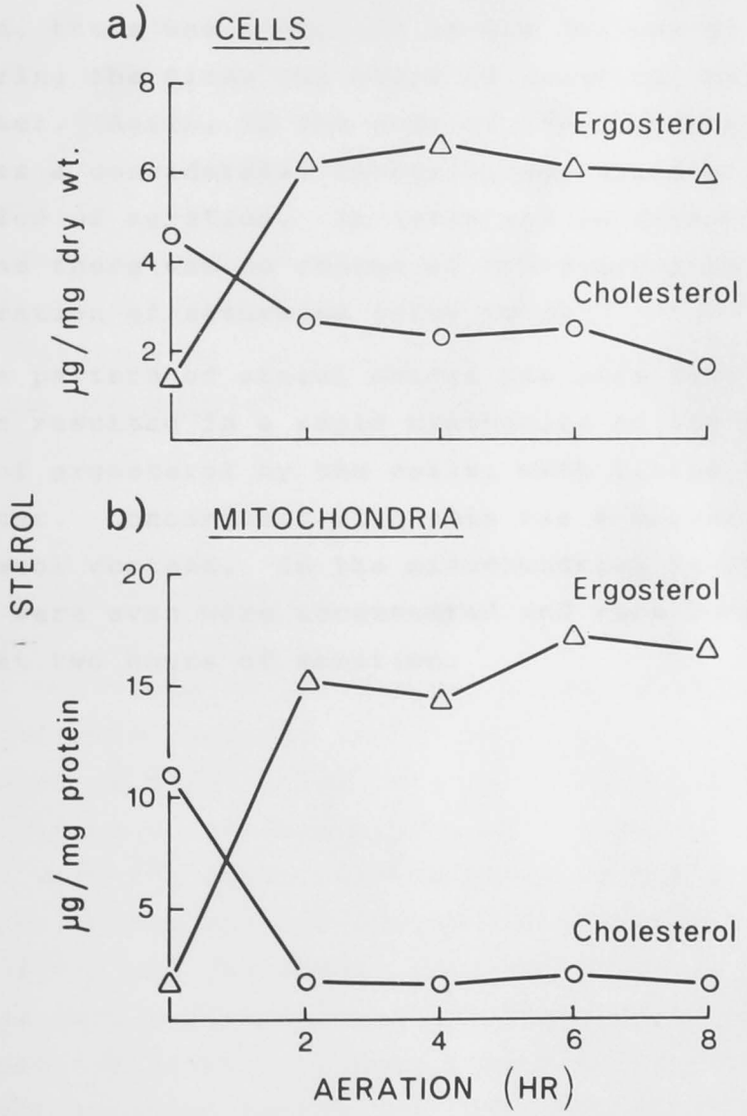

a) CELLS
60
40
20
Linoleic
Oleic
Palmitoleic
UNSATURATED FATTY ACID
µg/mg dry wt.
b) MITOCHONDRIA
200
100
Linoleic
Oleic
Palmitoleic
µg/mg protein
2
4
6
8
TIME (HR)

Figure 5-7



Sterol content of cells, and of mitochondria from these cells, grown anaerobically on linoleic acid and cholesterol and aerated in the absence of these lipids. Experimental details are given in Figure 5-6.

During aeration there was little significant change in the total amount of linoleic acid found in the cells. There was however, considerable synthesis of oleic and palmitoleic acids, though less extensive than that seen during aeration of lipid-depleted cells (Table 5-6). In the mitochondrial fraction, there was a decline in the content of linoleic acid during the first two hours of aeration, but little change thereafter. Again, in the case of the mono-unsaturated acids there was a considerable increase, approximately linear over the period of aeration. In cells and in mitochondrial fractions there was no change of consequence in the concentration of saturated fatty acids.

The pattern of sterol change was more substantial. Aeration resulted in a rapid synthesis, in the first two hours, of ergosterol by the cells, with little further change thereafter. Concomitant with this was a slower decline in cholesterol content. In the mitochondrial fraction, the changes were even more accentuated and were complete within the first two hours of aeration.

DISCUSSION

In this chapter effects on lipid synthesis that appear to be the consequence of the inhibition of protein synthesis have been described. Three of the lipids examined, unsaturated fatty acids, ergosterol, and ubiquinone, have a common feature in that in yeast all require molecular oxygen for their synthesis (Andreasen and Stier, 1953, 1954; Tchen and Bloch, 1957; Bloomfield and Bloch, 1960; Rudney and Raman, 1966). A fourth lipid class examined, phospholipid, also appears to require oxygen, at least for maximal synthesis, but this may be an indirect effect arising from a requirement for the unsaturated fatty acid component of the phospholipid. All of the above lipids are components of mitochondrial membranes.

1. THE INDUCED SYNTHESIS OF LIPIDS AND ENZYMES

The behaviour of cells grown anaerobically under two conditions were compared in the aeration system. The development of respiration, the formation of the aerobic cytochromes, and the development of ATPase activity during aeration were not radically different in the two anaerobic cell types. This contrasts with the results of previous workers (Hebb and Slebodnik, 1958; Morpurgo *et al.* 1964) who found that lipid-depleted cultures showed a substantially slower rate of adaption. Most likely the differences are due to the extended periods of anaerobic growth used by the earlier workers; this leads to loss of cell viability in lipid-poor cultures (Chapter IV).

Anaerobic cells grown with lipid supplements, and derived promitochondrial fractions, contain lipid in amounts that approach those found in aerobically-grown cells. Because of this the further synthesis of unsaturated fatty acids and ergosterol induced by aeration is relatively small. In contrast there are large increases in the levels of these

lipids on aeration of the lipid-depleted cells. The induction of ubiquinone follows similar kinetics in both types of cell. The rapid lag-free synthesis of lipids, cytochromes, ATPase (and other enzymes, Vary *et al.* 1970), and the rapid development of respiration, as well as the rapid re-establishment of protein synthesis (Chapter IV), attest to the viability of the lipid-depleted cultures used in these experiments, regardless of their lack of protein and RNA synthetic capacity after anaerobic growth.

2. *IN VIVO* EFFECTS OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON THE INDUCED SYNTHESIS OF LIPIDS: THE COUPLING OF THE MITOCHONDRIAL AND CYTOPLASMIC PROTEIN-SYNTHESISING SYSTEMS

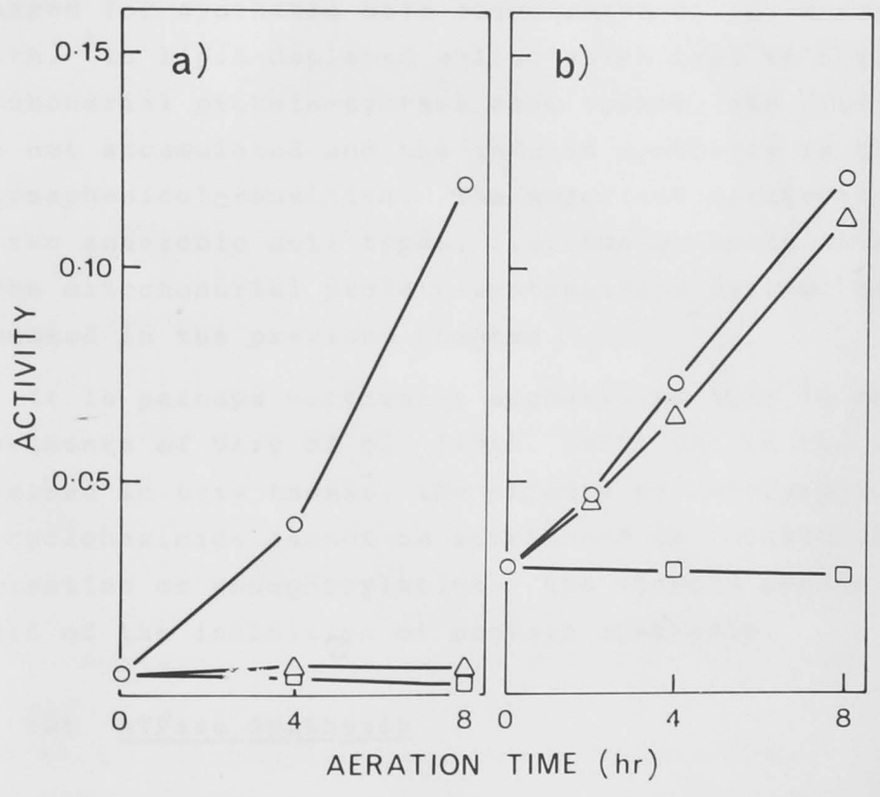
The results are best discussed with those obtained by Vary *et al.* (1969, 1970) on mitochondrial enzyme synthesis, and so a brief summary is included here, with succinate dehydrogenase (SDH) as an example. Figure 5-8 shows the time-dependent synthesis of SDH during the aeration of cells grown anaerobically without lipids (figure 5-8a) and with lipid supplements (figure 5-8b). The induced synthesis of SDH is completely sensitive to cycloheximide during aeration of both cell types. Chloramphenicol inhibits the induced synthesis only in lipid-depleted cells. The induced synthesis of malate dehydrogenase and fumarase can be similarly differentiated.

In interpreting these results it has been proposed that:

(i) mitochondrial enzymes such as SDH are synthesised on the cytoplasmic ribosomes; hence their synthesis is always sensitive to cycloheximide;

(ii) product(s) of the mitochondrial protein-synthesising system are also required for these syntheses, i.e. there is a 'coupling' between the two protein-synthesising systems;

Figure 5-8



Development of succinate dehydrogenase activity on aeration of cells grown anaerobically (a) without or (b) with lipid supplements. Aeration in the absence of antibiotics (O—O) aeration in the presence of chloramphenicol, 4 mg/ml, (Δ—Δ), and aeration in the presence of cycloheximide, 10 µg/ml, (□—□). Succinate dehydrogenase activity is expressed as µmoles substrate oxidized/min/mg protein.

Results are those of Dr. M. Lowdon

(iii) lipid-supplemented anaerobic cells have an active mitochondrial protein-synthesising system, and are therefore able to accumulate the required product(s) anaerobically, while lipid-depleted anaerobic cells lack such a system and therefore cannot accumulate the product(s).

Proposals (ii) and (iii) above predict the result that during aeration (under non-growing conditions) of lipid-supplemented cells, the synthesis of SDH is insensitive to chloramphenicol as the chloramphenicol-sensitive product(s) required for synthesis have accumulated during anaerobic growth. In lipid-depleted cells, which lack an active mitochondrial protein-synthesising system, the product(s) have not accumulated and the induced synthesis is therefore chloramphenicol-sensitive. The important distinction between the two anaerobic cell types, i.e. the presence or absence of the mitochondrial protein-synthesising system, has been discussed in the previous chapter.

It is perhaps worthwhile emphasizing that in the experiments of Vary *et al.* (1969, 1970) and in the experiments described in this thesis, the effects of chloramphenicol and cycloheximide cannot be attributed to inhibition of respiration or phosphorylation: the effects appear to be the result of the inhibition of protein synthesis.

(a) ATPase Synthesis

As outlined in Chapter I, the ATPase complex has been considered as at least two components, the soluble ATPase enzyme (F_1) which is cold-labile and oligomycin-insensitive, and another component which confers cold-stability and oligomycin-sensitivity. The latter component has been denoted F_c (Bulos and Racker, 1968) and called the oligomycin-sensitivity conferring protein, OSCP, by MacLennan and Tzagoloff (1968). In this study the F_1 component has been assayed. The oligomycin-sensitivity has been tested to estimate the relative proportion of 'free' and membrane-bound F_1.

The experiments can be considered with those of Tzagoloff (1969a,b), obtained from an examination of the effects of chloramphenicol and cycloheximide on ATPase (F_1) synthesis during glucose derepression. Cycloheximide inhibited the synthesis of ATPase (F_1) during both derepression (Tzagoloff, 1969b) and oxygen induction. These results are thus interpreted to mean that mitochondrial ATPase (F_1) is synthesised on cytoplasmic ribosomes. Also, Kovac and Weissova (1968) and Schatz (1968) have found that F_1 is synthesised in petite cells, implying that the formation of this enzyme is independent of the function of the mitochondrial protein-synthesising system. However during aeration of lipid-supplemented anaerobes, cycloheximide did not prevent an increase in the mitochondrial content of F_1, although the increase in whole homogenate activity was prevented. It appears that this is the consequence of a redistribution of F_1 present in the anaerobic cell.

The effects of chloramphenicol on ATPase (F_1) synthesis are most like those exerted on the synthesis of succinate dehydrogenase, and fumarase (Vary *et al.*1970), as the inhibition is seen during aeration of lipid-depleted cultures but not during aeration of lipid-supplemented cultures. It is possible therefore that product(s) of the mitochondrial protein-synthesising system are required for ATPase (F_1) synthesis. After aeration of lipid-depleted cultures in the presence of chloramphenicol the ATPase present is largely oligomycin-insensitive. It appears that under these conditions chloramphenicol inhibits the synthesis of the oligomycin-sensitivity conferring factor. These results, with those of Kovac and Weissova (1968) and Schatz (1968) on the ATPase in petite mutants, as well as the results of Tzagoloff (1969b), indicate that the OSCP is synthesised by the mitochondrial protein-synthesising system.

However the ATPase system is more complex, consisting of the soluble ATPase (F_1), the sensitivity factor (F_c or OSCP) and other component(s),possibly lipoprotein(s) of

the inner mitochondrial membrane (Bulos and Racker, 1968; Tzagoloff, 1970). The recent experiments of Tzagoloff (1970) suggest that the OSCP is synthesised on cytoplasmic ribosomes, like F_1, so the above results may have to be interpreted to mean that the other component(s) of the ATPase system are synthesised by the mitochondrial protein-synthesising system.

The lower ATPase activity in lipid-depleted anaerobic cells, compared with lipid-supplemented anaerobic cells, raises the question of the effect of lipids on ATPase levels, i.e. whether this is an effect on activity or synthesis. ATPase is an 'allotopic' enzyme (Racker and Bruni, 1968) in that when bonded to inner mitochondrial membrane its properties are changed. It has been shown (Bruni, Pitotti, Contessa, and Palatini, 1971, and references therein) that phospholipids strongly affect the sensitivity of ATPase to inhibitors. It is proposed that these phospholipids are involved at the site of inhibition (Bruni *et al.* 1971). However no structural or functional role can be assigned to phospholipid in the F_1 component of ATPase as purified preparations of F_1 contain no phospholipid (Penefsky and Warner, 1965; Racker, Tyler, Estabrook, Conover, Parsons, and Chance, 1965). It may be that the lower ATPase level in depleted cells is a consequence of the loss of protein synthetic capacity in these cells, and the turnover of the enzyme.

(b) Ubiquinone Synthesis

The fact that at least partial synthesis of ubiquinone, ergosterol, and unsaturated fatty acids occurs in the presence of cycloheximide or chloramphenicol indicates that the enzymes for these pathways are present in the anaerobic cell, and that the major restriction on their synthesis is lack of oxygen. The example of ubiquinone is particularly interesting as it is specifically located in the mitochondrion and is considered to function as an electron carrier in the respiratory chain rather than as a generally distributed

membrane structural component (see discussion in Chapter I). It has already been shown that the synthesis of ubiquinone is controlled by catabolite repression and oxygen tension, and in these respects behaves as a typical mitochondrial respiratory chain component, regardless of its site of synthesis (Chapter III). Moreover, the normal synthesis of ubiquinone in derepressed petites implies that the mitochondrial protein-synthesising system is not involved. Because of this it might be expected that chloramphenicol would not cause inhibition of ubiquinone biosynthesis, unless it also increased catabolite repression.

Ubiquinone synthesis during aeration of lipid-supplemented anaerobes is unaffected by chloramphenicol, even though the synthesis of cytochromes $\underline{aa}_3$ and $\underline{b}$ is almost completely inhibited. These results are consistent with those reported in Chapter III, and indicate that ubiquinone synthesis can be completely dissociated from the synthesis of other respiratory chain components. On the other hand the synthesis of ubiquinone during aeration of lipid-depleted anaerobes is partially sensitive to chloramphenicol. Cycloheximide causes a partial inhibition in both types of anaerobic cell. Partial inhibitions are difficult to rationalize, and are perhaps best considered with the inhibitor effects on the other lipids. It is interesting to note that the syntheses of ubiquinone and cytochrome $\underline{c}$ were not dissociated by any of the conditions used, with the exception of aeration in the presence of cycloheximide.

When the effects of chloramphenicol and cycloheximide are examined at the mitochondrial level it is apparent that these antibiotics decrease the specific content and the proportions of ubiquinone recovered in this fraction. Again, it is difficult to interpret these effects unequivocally because the organelles assembled in the presence of the inhibitors may be more fragile and thus more susceptible to disruption during isolation. Even so, the results show that it is possible to attain considerable incorporation of

ubiquinone into mitochondria, even in the absence of particulate cytochrome synthesis and assembly. It appears that the assembly as well as the synthesis of ubiquinone can be dissociated from that of other members of the respiratory chain.

(c) Synthesis of Ergosterol, Unsaturated Fatty Acids, and Phospholipid

As discussed in Chapter I, the syntheses of ergosterol, unsaturated fatty acids, and phospholipid have been examined in the hope that they would provide an indication of the synthesis and assembly of the structural lipids of membranes. It is realized that there may be no ultimate distinction between structural and functional (catalytic) lipids, and that a lipid may have both roles; in the absence of evidence as to a specific catalytic role for these lipids in oxidative phosphorylation, they will be referred to as structural lipids in contrast to ubiquinone.

As indicated earlier, the higher initial levels of lipid in lipid-supplemented anaerobes largely obscured any potential antibiotic effects on lipid synthesis during aeration of this type of cell. During aeration of lipid-depleted cells, there is extensive synthesis of ubiquinone, unsaturated fatty acids, and ergosterol, and incorporation of these components into the mitochondrial fraction. The respiratory enzymes (particulate and soluble) also show large increases. Phospholipid levels increase by a relatively small amount. Again, the effects of the antibiotics on the induced enzyme and lipid syntheses are best considered together, and these effects are summarized in Table 5-14: succinate dehydrogenase activities are used in the table but the effects extend to malate dehydrogenase and fumarase at least.

During aeration of lipid-depleted cells in the presence of chloramphenicol, all the proteins normally synthesised are inhibited, with the exception of cytochrome c. The sterol

TABLE 5-14

EFFECTS OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON MITOCHONDRIAL DEVELOPMENT

Growth Conditions	cytochromes			SDH	Respiration	UQ	PL	UFA	ERG	Cristae
	aa_3	b	cc_1							
Anaerobic, lipid-depleted										
Aerated 0 hr	-	-	-	-	-	-	++	+	-	-
Aerated 8 hr	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++
Aerated 8 hr and CAP	-	+	+++	-	-	++	+++	++	++++	+
Aerated 8 hr and CYC	-	-	-	-	-	+	.	++	++	-
Anaerobic, lipid-supplemented										
Aerated 0 hr	-	-	-	+	-	-	+++	+++	++	-
Aerated 8 hr	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Aerated 8 hr and CAP	-	+	+++	++++	-	++	.	.	.	++
Aerated 8 hr and CYC	-	-	-	-	-	+	.	.	.	-
Aerobic	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

Cells were grown and aerated as described. Values are indicated relative to the levels found in aerobic cells, and are taken from tables and figures presented earlier. A dash means that the level is very low or zero; dots represent values not measured. Abbreviations used : SDH, succinate dehydrogenase; UQ, ubiquinone; PL, phospholipid; UFA, unsaturated fatty acid; ERG, ergosterol.

content of the mitochondrial fraction appears also to be unaffected by this antibiotic. Under these conditions ubiquinone and unsaturated fatty acid synthesis and integration are partially inhibited. Cycloheximide causes a partial inhibition of the synthesis of lipids, and complete inhibition of the formation of cytochromes, respiratory enzymes, and respiration.

In general terms, the results of the inhibitor studies show that the interlock between the syntheses of different mitochondrial proteins is not rigid. Similarly the interlock between protein synthesis and lipid synthesis is not rigid: conditions can be defined where inhibition of the mitochondrial protein-synthesising system does not affect the synthesis of mitochondrial lipids or some mitochondrial proteins. However cycloheximide under any of the conditions examined inhibited the synthesis of these lipids, at least partially, and chloramphenicol was inhibitory during aeration of lipid-depleted anaerobes. These results indicate some degree of coupling between the two protein-synthesising systems and the systems responsible for lipid synthesis. The actual extent of this coupling appears to be dependent on the presence or absence of added lipids in the anaerobic phase of growth. By analogy with the enzyme results of Vary *et al.*(1969, 1970), discussed above, this would indicate that the presence or absence of a functional mitochondrial protein-synthesising system at the start of aeration is an important determinant of the extent of coupling observed. This hypothesis is developed further in the following two chapters.

It is worth noting that the unsaturated fatty acid content of both whole cells and mitochondria is inhibited by chloramphenicol to about the same extent. This indicates that the effect of chloramphenicol on the synthesis of this lipid is a generalized one, i.e. mitochondrial lipids are not specifically affected.

3. LIPID SYNTHESIS AND RESPIRATORY ADAPTION

It has been proposed that the mitochondrial protein-synthesising system synthesises product(s) required for the synthesis of some mitochondrial proteins. If this is true then factors that affect the activity of the mitochondrial protein-synthesising system, for example lipid depletion under anaerobic growth conditions, will affect the synthesis of enzymes controlled by this system. The observations discussed in this and the previous chapter support this hypothesis. However the importance of lipids during the aeration process still remains largely undefined.

Several groups of workers (see introduction to this chapter) have suggested that lipid synthesis is related to respiratory development. The results presented in this study show at least that in the wild-type organism there is a complex set of relationships between lipid synthesis (or exogenous supply) and the synthesis of mitochondrial proteins during aeration, whether these are of cytoplasmic or mitochondrial origin. It is important to distinguish between effects of lipids on protein synthesis and effects of lipids on enzyme activity. For example, it is possible that the lack of enzyme synthesis during aeration of depleted cells in the presence of chloramphenicol is due to an inhibition of lipid synthesis. This has been tested by Vary *et al.* (1970). These workers have shown that the chloramphenicol inhibition is not removed by the addition of lipids to the aeration medium, i.e. lipids cannot simply substitute for the postulated product(s) of mitochondrial protein synthesis. Further evidence relating to this point has been presented in the previous chapter. It was found that the rapid re-establishment of the activity of the mitochondrial protein-synthesising system on aeration of depleted cells could, in some cases, occur without <u>further</u> lipid synthesis. This may suggest that lipid synthesis and the re-establishment of mitochondrial protein synthesis can be independent events, i.e. protein synthesis may not be coupled <u>to</u> lipid synthesis

in some circumstances. However in view of the coupling of lipid synthesis to protein synthesis, this suggestion has to be carefully considered. This is perhaps best attempted in the following chapters.

4. OTHER STUDIES ON THE INTERREGULATION OF PROTEIN AND LIPID SYNTHESIS

Recently several reports have been published on the problems discussed above. In studies with rat liver it has been postulated that protein and lipid components of cytochrome oxidase (Schiefer, 1969), and of inner and outer membrane of mitochondria (Beattie, 1969a), are synthesised synchronously. It was found that inhibition of protein synthesis with cycloheximide resulted in inhibition of lipid synthesis, suggesting that lipid and protein synthesis in these membranous components of rat liver were subject to considerable interregulatory control. But, as previously discussed, it is extremely difficult to interpret *in vivo* mammalian studies.

Mycoplasma laidlawii provides a good experimental system for studies on membrane biogenesis, as the plasma membrane is easily purified (there is no cell wall) and in contrast to mitochondria and chloroplasts there is but a single membrane system. These organisms require a source of unsaturated fatty acid for growth (Rodwell, 1968). The added unsaturated fatty acid is incorporated into the membrane phospholipid, so that it may comprise more than 90 per cent of the fatty acid of this fraction (Rodwell, 1968). Kahan and Razin (1969) have studied the incorporation of ^{3}H-oleic acid and ^{14}C-phenylalanine into the membranes of *M. laidlawii*. They found that when membrane protein synthesis was inhibited by chloramphenicol the lipid incorporation was not affected, so that the density of the membranes decreased. These authors conclude that in this system the assembly of lipid and protein components of membranes are not necessarily coupled.

Similar conclusions have been reached by Mindich (1970a, b) in a study of lipid and protein synthesis in glycerol-requiring mutants of *Bacillus subtilis*. When these mutants were deprived of glycerol they ceased net synthesis of phospholipid, yet the incorporation of labelled amino acids into membrane protein continued. In this respect, this study is complementary that of Kahane and Razin (1969). Mindich (1970b) also concluded that the incorporation of lipids and proteins into membranes can be uncoupled so that the composition of the membrane changed.

In *Euglena gracilis*, chloramphenicol inhibits chlorophyll synthesis and the formation of certain chloroplast proteins during light-induced greening; the synthesis of galactolipids, which are specific to this organelle, is partially inhibited under these conditions (Bishop and Smillie, 1970). The change in fatty acid composition of the total lipids of *Euglena* that accompanies greening are rather complex, but chloramphenicol tends to inhibit these changes. These authors also found that the effect of cycloheximide is dependent on the concentration used, but at concentrations that inhibited chlorophyll synthesis, inhibition of galactolipid synthesis was almost complete. Cycloheximide also inhibited the changes in the unsaturated fatty acid composition that accompany the greening process.

5. CONVERSION OF PROMITOCHONDRIA INTO MITOCHONDRIA

The experiments described in which cell and promitochondrial lipids were preloaded in the anaerobic phase of growth with linoleic acid and cholesterol make an interesting comparison with those described recently by Plattner, Salpeter, Saltzgaber, and Schatz (1970). These workers found that promitochondrial protein, labelled with radioactive amino acid in the anaerobic phase, accounted for about 50 per cent of the protein found in the mitochondria after 8 hours of aeration. Similarly, in the present study

about one half of the linoleic acid originally present in the promitochondria persisted during aeration, although this value may also reflect the rate at which unsaturated fatty acid is recycled though membranes and pools in the cells during aeration. Metabolic transformation is unlikely for the reasons discussed earlier. Taken together, these results would seem to indicate that a large proportion of certain proteins and lipids is conserved during organelle development initiated by aeration, i.e. there appears to be a precursor-product relationship between promitochondria and mitochondria.

The other lipid class examined, sterol, does not seem to be conserved during mitochondrial development. There was a rapid and almost complete replacement of the cholesterol in promitochondrial membranes by ergosterol in the early stages of aeration. The reason for this is not apparent.

CHAPTER VI

STUDIES ON THE CO-ORDINATION OF SYNTHESIS OF ENZYMES AND LIPIDS DURING RESPIRATORY ADAPTION. II. THE USE OF MUTANTS

INTRODUCTION

1. AN ALTERNATIVE APPROACH

During the biogenesis of mitochondria it is likely that the formation of the lipid-protein complexes, which constitute the membranes and enzyme systems of this organelle, involves some degree of co-ordination between lipid and protein synthesis. In previous sections of this study the applicability of the anaerobic/aerobic transition has been discussed. It has been suggested that there is considerable coupling between the mitochondrial and cytoplasmic protein-synthesising systems on the one hand and the lipid-synthesising systems on the other during the formation of mitochondria from promitochondria. This postulate was based on a study of the effects of chloramphenicol and cycloheximide on the induced synthesis of mitochondrial enzymes and lipids. However, results obtained from inhibition studies can only be interpreted on the basis of their mode of action, which may be incompletely understood and can be complicated by side effects. Ideally, the conclusions from inhibitor studies should be confirmed by other means; possible alternate means are considered in this chapter.

In yeast, a number of mutants are available in which there are specific blockages in either lipid or protein synthesis. Of course, mutations which inactivate cytoplasmic protein synthesis, or mitochondrial protein synthesis in more advanced organisms, are lethal. But in facultative anaerobes like *S. cerevisiae* mutations which affect mitochondrial protein synthesis are not lethal, and cells can grow and divide if fermentable substrate is provided.

Conversely, the use of mutants with altered lipid synthesis is complicated by the fact that no mutants in which the synthesis of mitochondrial lipids is specifically affected have been isolated. Nevertheless, mutants blocked in some part of general lipid synthesis have been isolated, and in *S. cerevisiae* an unsaturated fatty acid requiring mutant has been described by Resnick and Mortimer (1966).

2. THE CYTOPLASMIC PETITE MUTATION

Many of the characteristics of the neutral cytoplasmic petite mutation have been reviewed in the General Introduction to this thesis. The primary cause of the mutation appears to be a change in the mitochondrial DNA, in many cases resulting from a loss of this genetic material. The most obvious phenotypic result is a loss of respiration. More recently the biochemical changes in petite mitochondria have been better defined. In addition to the loss of ability to synthesise the particulate cytochromes and the loss of respiration, the formation of the ATPase complex is affected. It has been found that even though oxidative phosphorylation is not possible in these mutants, they are still affected by inhibitors of oxidative phosphorylation (Kovac and Weissova, 1968),the implication being that the partial reactions of oxidative phosphorylation are still functional. This conclusion is indirectly supported by the results of Groot *et al.* (1971) who showed that promitochondria from lipid-supplemented anaerobic cells contained a Pi-ATP exchange reaction, even though of course no respiration was evident. The conversion of ATP to other phosphorylated intermediates may be essential to the overall economy of the cell. Kovac and Weissova (1968) and Schatz (1968) have reported that petites contained ATPase (F_1) although sensitivity to oligomycin had been lost. As discussed in the previous chapter, this has been interpreted to mean that the synthesis of a factor responsible for sensitivity is inhibited by the mutation, while F_1 synthesis is unaffected and is preserved in the oxidative phosphorylation system.

Much more relevant to the present study are reports (Wintersberger, 1967; Kuzela and Grecna, 1969; Schatz and Saltzgaber, 1969a) that petite cells contain no active mitochondrial protein-synthesising system. The petite mutant therefore offers an opportunity to test in a different way some of the possibilities raised in the previous chapter. If the partial inhibitions of lipid synthesis by chloramphenicol and cycloheximide that were observed in wild-type cells are due to a coupling between the two protein-synthesising systems and lipid synthesis, then the absence of a mitochondrial protein-synthesising system should affect the response of the cells to these inhibitors. It would be expected that the petite would be unable to form products which require the activity of the mitochondrial protein-synthesising system. As well, any control mechanism which depended directly or indirectly on this system would be affected.

3. THE UNSATURATED FATTY ACID AUXOTROPH (KD115)

The other mutant that has been used, denoted KD115, is one of a class of mutants that have been isolated by Resnick and Mortimer (1966). These require unsaturated fatty acid for growth under aerobic, as well as anaerobic, conditions. In KD115 the requirement for unsaturated fatty acid results from a nuclear mutation which leads to the absence of Δ^{9}-desaturase activity (Keith, Resnick and Haley, 1969) although the mutant is still able to synthesise saturated fatty acids.

The availability of this mutant from Mortimer's laboratory suggested that a complementary approach to that outlined for the petite might be possible. As mentioned above, the difficulty is that the unsaturated fatty acid requirement is not a specifically mitochondrial characteristic, so if the mutant were to be used most appropriately conditions under which mitochondrial lipid composition are selectively affected should be defined. This has been a major difficulty with experiments involving the use of KD115.

RESULTS

1. EFFECT OF UNSATURATED FATTY ACID SUPPLY ON THE DEVELOPMENT OF RESPIRATION IN KD115

The unsaturated fatty acid and sterol levels and the respiration rate of KD115 can be decreased in the same way as the wild-type and petite strains, i.e. by anaerobic growth in a lipid-poor medium. In the following experiments we have decreased the levels of mitochondrial respiratory components and lipid in this way, and then measured the formation of respiratory enzymes and cytochromes, and the development of respiration, induced by aeration of these anaerobically grown cells. Aeration was carried out in the presence and absence of Tween 80 (a water-soluble source of unsaturated fatty acid). It was hoped that any of the mitochondrial components that required unsaturated fatty acid for their formation would be detected in this way.

KD115 cells, grown anaerobically without lipid supplements, had only traces of respiratory activity (Table 6-1). Aeration of these cells in the presence of Tween 80 resulted in the rapid development of mitochondrial function, as indicated by the development of respiration to levels that approximated those found in batch cultures grown aerobically with unsaturated fatty acid supplement. Aeration in the absence of unsaturated fatty acid resulted in poor respiratory development (Table 6-1). This inhibition of respiration was similar in magnitude to that seen when cells were aerated in the presence of unsaturated fatty acids and chloramphenicol.

Table 6-1 also shows the unsaturated fatty acid levels of KD115 cells grown under the conditions described. Cells grown anaerobically without added lipids were considerably depleted compared with cells grown aerobically with Tween 80. Aeration of these depleted cells in the absence of Tween 80 caused a further slight decrease in the unsaturated fatty acid content of the cells, while aeration

TABLE 6-1

EFFECT OF GROWTH AND AERATION CONDITIONS ON RESPIRATION AND UNSATURATED FATTY ACID CONTENT OF KD115

Growth Conditions	Respiration[1]	Unsaturated fatty acid (mg/g cells)
anaerobic	7	7
aerobic, plus Tween 80	88	25
anaerobic; aerated 10 hr (plus Tween 80)	110	36
anaerobic; aerated 10 hr (without Tween 80)	30	4.5
anaerobic; aerated 10 hr (plus Tween 80, plus CAP)	9	26
anaerobic; aerated 10 hr (without Tween 80, plus CAP)	6	5

Cells were grown aerobically with Tween 80, or anaerobically (22 hr) without Tween 80. Anaerobic cells were aerated under the conditions described. Tween 80, where added, was present at 1 ml/per 200 ml medium; chloramphenicol (CAP) was present at 4 mg/ml (9 mM).

1. Respiration (ng at Oxygen/min/mg cells) is given as that sensitive to 1 mM KCN. Cyanide-insensitive respiration in all cells was 2-5 ng at 0/min/mg cells.

* * * * * *

in the presence of Tween 80 resulted in the uptake of unsaturated fatty acids to essentially normal cellular levels. Chloramphenicol had little effect on this incorporation of unsaturated fatty acids.

The results presented above suggest that a source of unsaturated fatty acid is required for normal respiratory development. The chloramphenicol inhibition indicates that the activity of the mitochondrial protein-synthesising system is required as well.

2. INHIBITION OF CYTOCHROME SYNTHESIS BY UNSATURATED FATTY ACID RESTRICTION IN KD115 DURING AERATION

Figure 6-1a, curve A, shows the whole cell cytochrome spectrum of lipid-depleted anaerobic KD115 cells, with broad peaks at 505, 555 and 585 nm. When these lipid-depleted cells were aerated, the extent of cytochrome formation was found to depend on the presence of Tween 80 in the aeration medium. With no unsaturated fatty acid in the medium cytochrome induction was very limited (curve B). But in the presence of Tween 80 distinct cytochrome $\underline{c}$ + $\underline{c}_1$ (550 nm) and cytochrome $\underline{b}$ (560 nm) peaks and an indistinct cytochrome $\underline{aa}_3$ peak (604 nm) became apparent.

When KD115 was grown anaerobically in the presence of unsaturated fatty acid and sterol a different type of anaerobic spectrum resulted (figure 6-1b, curve A), with a broad peak centred around 560 nm. (Both types of anaerobic spectrum are similar to the wild type *S. cerevisiae* when grown under similar conditions; Wallace, Huang and Linnane, 1968; Chapter V). Aeration of this lipid-supplemented anaerobe in the absence of Tween 80 resulted in the formation of distinct cytochrome $\underline{aa}_3$, $\underline{b}$ and $\underline{c}$ + $\underline{c}_1$ peaks (figure 6-1b, curve B). However, these aerobic peaks were augmented when Tween 80 was included in the aeration medium.

3. EFFECT OF UNSATURATED FATTY ACID CONTENT ON THE SYNTHESIS OF RESPIRATORY ENZYMES IN KD115

The results presented in the above two sections show that unsaturated fatty acid is required for the development of respiration and for cytochrome synthesis. Further, the experiments on cytochrome synthesis presented above, and other experiments on the induction of respiration, suggested that the extent of respiratory development during aeration of anaerobic lipid-depleted KD115 cells in the absence of added unsaturated fatty acid was dependent on the amount of

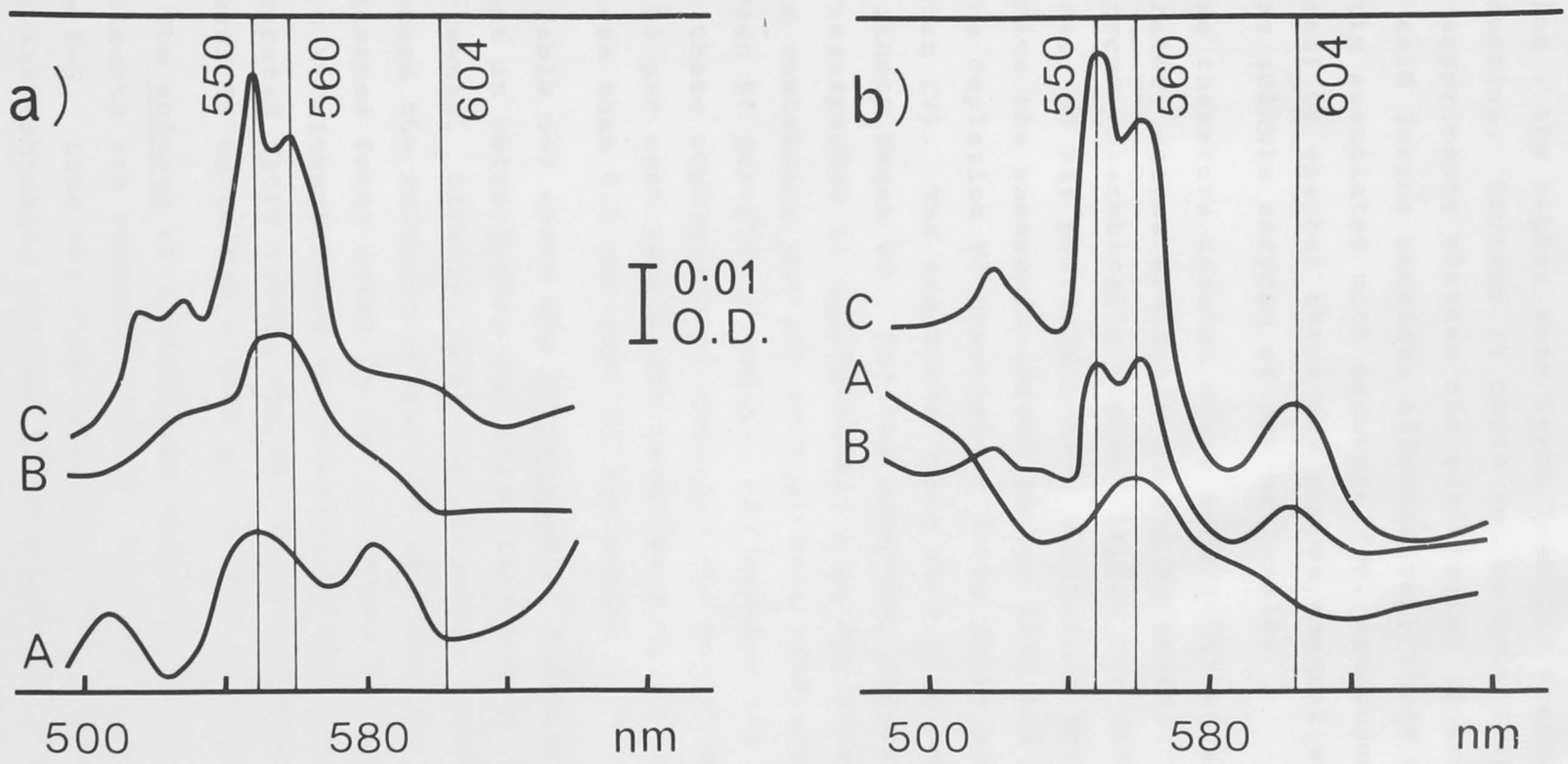


Figure 6-1

Whole cell cytochrome spectra of KD115 grown anaerobically (a) without lipid supplements and (b) with lipid supplements, (curves A), then aerated for 8 hr without (curves B) or with (curves C) Tween 80 in the aeration medium. Where present in the aeration medium, the concentration of Tween 80 was 5 ml/l.

this lipid present in the anaerobic cell at the start of aeration : the higher this level then the greater the extent of induction. However it could not be established from these experiments whether the restriction of unsaturated fatty acid during aeration affected only those enzymes directly associated with membrane (eg. cytochromes, flavoproteins) or whether there was a more general effect, to include soluble enzymes of the organelle.

We therefore carried out a more detailed analysis of respiratory enzyme synthesis during aeration. KD115 cells were grown anaerobically without lipids for different periods. It has previously been shown that the more extensive the anaerobic growth period then the more extreme was the depletion of unsaturated fatty acids and ergosterol (Chapter IV). The anaerobic cells were then aerated with and without Tween 80. For the aeration, anaerobic cells were resuspended at approximately 4 mg dry wt/ml in synthetic medium containing one per cent glucose with and without one ml Tween 80 per 200 ml medium, and aerated for 10 hours. Under these conditions of aeration the cells remain greater than 90 per cent viable and revertants to wild-type accounted for less than 0.5 per cent of the cells.

Table 6-2 shows the <u>proportion</u> of different fatty acids present in cells before and after aeration in two typical experiments. Clearly, when the anaerobic growth phase was prolonged the relative proportion (per cent by weight) of unsaturated fatty acids in the cells was decreased. Aeration for 10 hr without Tween 80 resulted in further decreases in unsaturated fatty acids, whereas cells aerated in the presence of Tween 80 contained a high proportion of these acids.

The <u>amounts</u> of unsaturated fatty acids in these two experiments are compared with the ergosterol content in Table 6-3. Like the wild-type and petite organisms, KD115 grown anaerobically without lipid supplements contained only small amounts of ergosterol. Where anaerobic growth was limited the synthesis of this lipid was little affected by

TABLE 6-2

FATTY ACID DISTRIBUTION BEFORE AND AFTER AERATION OF KD115

Aeration time (hr)	UFA in aeration medium	Extent of depletion[1]	Fatty acid distribution[2] (%, w/w)							
			$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{14:1}$	$C_{16:1}$	$C_{18:1}$
0	-	partial	4.8	6.8	11.9	40.0	6.8	0.0	3.7	26.4
	-	complete	11.8	9.7	10.4	44.1	8.1	0.0	1.5	14.4
10	-	partial	6.1	6.2	10.1	49.4	10.0	0.0	2.2	16.1
	-	complete	7.8	9.4	9.0	50.0	11.6	0.0	0.6	11.6
10	+	partial	0.7	1.5	3.6	28.1	6.2	0.8	7.3	51.8
	+	complete	2.0	2.6	6.2	24.6	6.0	1.0	7.4	50.3

1. 'Partial' depletion : cells were grown anaerobically without lipid supplements for 24 hr.
   'Complete' depletion : anaerobic growth phase extended to 37 hr.

2. Fatty acids are denoted by convention; number of carbon atoms : number of unsaturated bonds. Cells were aerated in synthetic media (defined in Chapter II); where present, unsaturated fatty acid (UFA) was added to the aeration medium as Tween 80 (5 ml/l).

TABLE 6-3

FATTY ACID AND ERGOSTEROL CONTENT OF KD115

Aeration time	UFA in aeration medium	extent of depletion	UFA (%,w/w)	UFA (mg/g cells)	ERG (mg/g cells)
0	-	partial	30.1	7.7	0.41
	-	complete	15.9	5.0	0.34
10	-	partial	18.3	5.0	3.8 (92)
	-	complete	11.6	4.0	2.4 (42)
10	+	partial	59.9	30.2	4.1
	+	complete	58.7	21.6	5.3

Experiments are those described in Table 6-2. Unsaturated fatty acid (UFA) and ergosterol (ERG) were measured. Values in parentheses are defined in Table 6-4.

* * * * * *

the absence of Tween 80 during the following aeration phase. When the lipid level of the anaerobe was further depleted by prolonged anaerobic growth, ergosterol synthesis was considerably lower in cells aerated in the absence of Tween 80, compared with those aerated in its presence.

Table 6-4 presents the activities of representative mitochondrial enzymes, and catalase, found in cells from the two experiments described above. It can be seen that the levels of all enzymes examined were lowest in the anaerobically-grown cells, and induction of these enzymes occurred when the cells were aerated. But the extent of enzyme induction varied depending on the extent of lipid depletion during anaerobic growth, and on the presence of Tween 80 during aeration. When the anaerobic cells were aerated in the presence of Tween 80 there were large increases in the enzyme levels in both experiments. In contrast, aeration without lipid supplement limited the

TABLE 6-4

INDUCTION OF ENZYMES DURING AERATION OF KD115 : EFFECT OF UFA

Aeration time (hr)	UFA in aeration medium	Extent of depletion	Malate dehydrogenase	Fumarase	Succinate dehydrogenase	Cytochrome oxidase	Catalase
			(μmoles substrate converted/min/mg protein)				
0	-	partial	0.98	0.090	0.025	0.005	0.26
	-	complete	0.92	0.082	0.023	0.005	0.23
10	-	partial	3.00(51)	0.150(31)	0.040(15)	0.096(68)	2.32(24)
	-	complete	1.10 (7)	0.105(12)	0.042 (6)	0.020(11)	1.34(17)
10	+	partial	5.00	0.292	0.125	0.141	8.96
	+	complete	3.50	0.270	0.317	0.136	6.77

Experiments are those described in Tables 6-2 and 6-3.

Enzymes activities were measured by Dr. M. Lowdon, after preparation of whole homogenates by the French press method. Values in parentheses are percentages expressed relative to the increase observed when unsaturated fatty acid (UFA) was present in the aeration medium.

extent of enzyme induction, with the decrease being most pronounced where lipid depletion was more complete. These results are summarized by the values in parentheses (Tables 6-3 and 6-4). It can be seen that, in the absence of added unsaturated fatty acid, the level of this lipid in the anaerobic cell prior to aeration apparently determined the extent of enzyme synthesis during aeration. There was no apparent difference between particulate and soluble enzymes in this respect. These results are consistent with those obtained from the spectral measurements and the respiration studies.

In the experiments above, catalase was assayed as an oxygen-inducible, non-mitochondrial enzyme, i.e. it was thought that it would provide a control to test the specificity of the effects of unsaturated fatty acid starvation on mitochondrial development. Table 6-4 shows that the extent of induction of catalase activity was also affected by unsaturated fatty acid deficiency. However the development of catalase activity depends on heme synthesis, as well as the synthesis of apoprotein, and part of the heme pathway is located in the mitochondria (Lascelles, 1964). The lack of catalase formation under lipid-deficient conditions may thus reflect a requirement for unsaturated fatty acid in the synthesis or activity of this mitochondrial portion of the pathway, so that catalase may not provide the most satisfactory control. Another possibility is that under conditions of lipid starvation there may be an increased glucose repression, for similar reasons to those discussed for the wild-type and petite (see Chapter III). All the enzymes measured are sensitive to catabolite repression.

On the other hand, the inhibition of catalase may indicate that the effect of unsaturated fatty acid starvation on enzyme synthesis is a general one, perhaps limiting the ability of the cell to form membrane-ribosome complexes for protein synthesis. In an effort to estimate general protein synthesis in the cells, and so test some of these possibilities, we have measured the incorporation of labelled leucine into protein.

4. THE EFFECT OF UNSATURATED FATTY ACID RESTRICTION ON PROTEIN SYNTHESIS DURING AERATION OF KD115

Because of the likely complication of catabolite repression, we have used in the following experiments an aeration procedure in which there was a progressive feed of glucose to the aeration flasks at rates which maintained free glucose at a constant low concentration, rather than the earlier procedure in which glucose was added batchwise at the start of aeration. Figure 6-2 shows the results of an experiment in which glucose was added at two different rates while lipid-depleted anaerobic KD115 cells were aerated under various conditions.

The higher glucose concentration repressed the development of respiration which occurred on aeration in the presence of Tween 80 (Fig. 6-2a, d), while the glucose concentration made little difference when chloramphenicol was present (Fig. 6-2b, e). When cells were aerated in the absence of Tween 80 (Fig. 6-2c, f) then the higher pump rate caused an accumulation of glucose in the medium. There appears to be a decreased utilisation of glucose under conditions of lipid starvation, an observation which supports the possibility that these conditions enhance catabolite repression and increases the necessity to use non-repressing conditions. The lower pump rate did not cause an accumulation of glucose in this type of aeration, and this rate has been used in the following experiments.

The effects of the presence of various amounts of unsaturated fatty acid (as Tween 80) on the development of respiration and the incorporation of ^{14}C-leucine into protein during the aeration of lipid-depleted anaerobic KD115 cells is shown in Figure 6-3. At levels of added unsaturated fatty acid less than 0.12 mg/ml the development of respiration was inhibited, as in the experiments described earlier. The incorporation of ^{14}C-leucine into protein was also restricted by the availability of lipid, although it

figure 6-2

Effect of glucose feed rate on the development of respiration and the glucose in the medium during aeration of anaerobically-grown, lipid-depleted KD115 cells. Glucose solution was pumped into the aeration medium (synthetic medium, as defined in Chapter II) at the rate of 5 1/3 ml/hr/200 ml culture: the glucose concentration of the feed media was 12.5 per cent (square symbols) or 25 per cent (circles). The aeration medium contained Tween 80 (5 ml/l) and Chloramphenicol, CAP. (4 mg/ml), as indicated on the diagrams. The 3 upper diagrams describe the glucose concentration in the various media, and the lower diagrams show the development of respiration in the corresponding media.

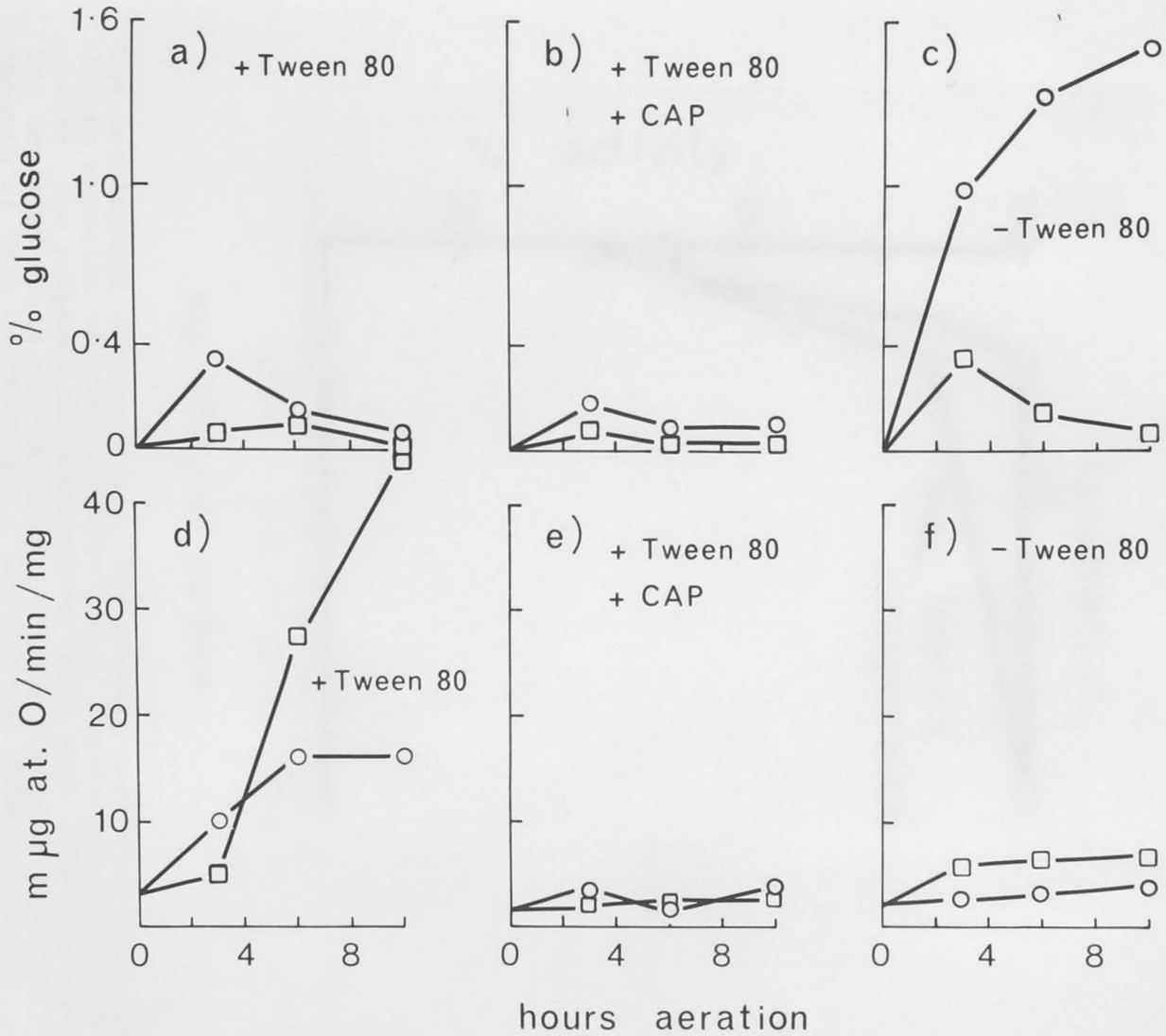
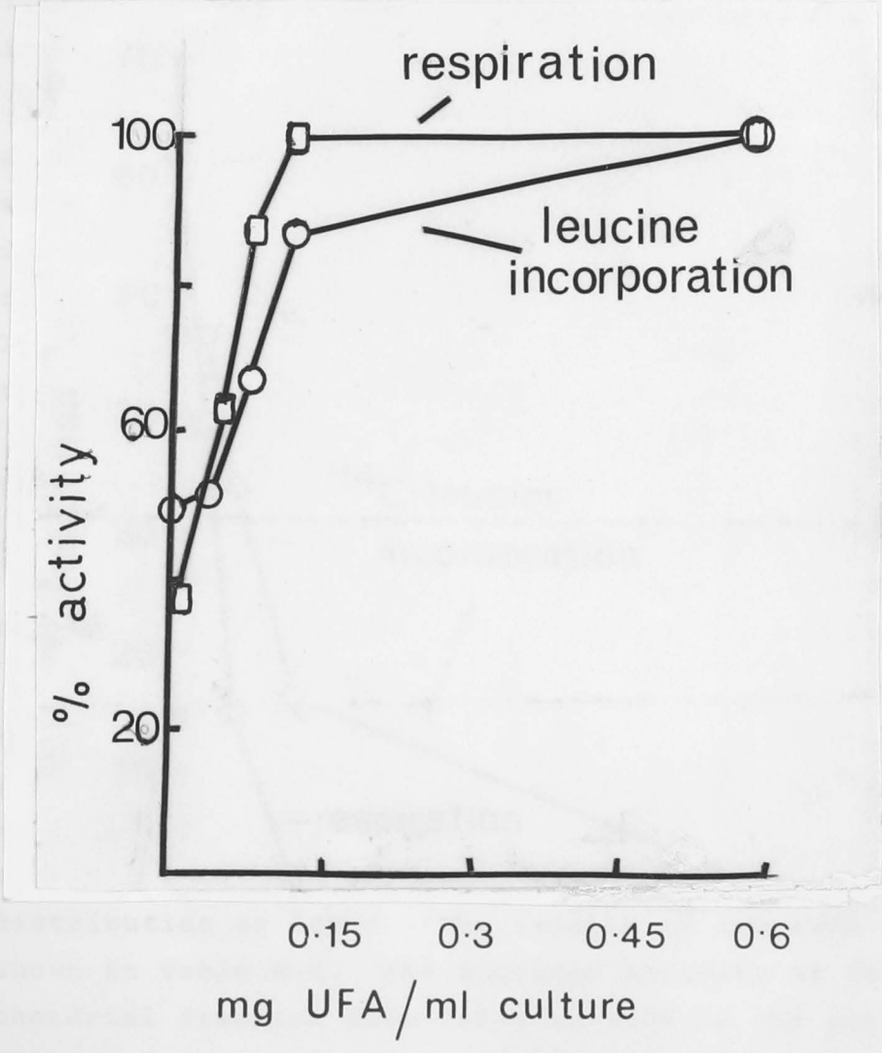

a)
+ Tween 80
b)
+ Tween 80
+ CAP
c)
- Tween 80
d)
+ Tween 80
e)
+ Tween 80
+ CAP
f)
- Tween 80
% glucose
m µg at. O/min/mg
1·6
1·0
0·4
0
40
30
20
10
0
4
8
hours aeration

Figure 6-3



The development of respiration and protein-synthetic activity during aeration of anaerobically-grown, lipid-depleted KD115 cells; the response to unsaturated fatty acid (UFA) addition. The depleted cells were aerated in the presence of various amounts of UFA(as Tween 80). Glucose was pumped into the culture as described in the text. Results are expressed as per cent activities relative to the activities found when 0.6 mg UFA/ml culture was used.

did not appear to be as strongly affected as respiratory development, indicating that there may have been some selective inhibition of respiration. It is unlikely that the inhibition of protein synthesis is due to restricted energy supply in the cells unable to develop normal coupled respiratory activity, as the cultures were provided with a continuous supply of fermentable substrate.

Typical incorporation rates of ^{14}C-leucine into protein at various stages of aeration are shown in figure 6-4. In the early period of aeration (Fig. 6-4a) there was considerable variation, however chloramphenicol either had no effect or stimulated slightly, while the lack of Tween 80 usually caused slight inhibition. The incorporation patterns for the latter stages of aeration were much more reproducible : both the presence of chloramphenicol in the aeration medium and the absence of Tween 80 led to a 60-70 per cent inhibition (Fig. 6-4b,c). If the inhibitory treatments were combined, i.e. aeration in the presence of chloramphenicol and the absence of Tween 80, there was no increase in the inhibition.

It thus appeared that inhibition of mitochondrial protein synthesis (by chloramphenicol) or the lack of unsaturated fatty acids both effected the same degree of inhibition of incorporation. This has been examined in more detail by fractionation of the cells after labelling and determining the distribution of label : the results of two such experiments are shown in Table 6-5. The specific activity of the mitochondrial fraction from cells aerated in the presence of unsaturated fatty acid was approximately twice that of the soluble protein. Omitting unsaturated fatty acids from the aeration medium caused a large inhibition (approximately 60 per cent) of the specific activity of both fractions, so that the ratio of labelling was only decreased slightly. However the addition of chloramphenicol decreased the ratio drastically and there was little preferential labelling of mitochondrial protein, although again there was a large inhibition in both cellular compartments. Apparently chloramphenicol inhibition

figure 6-4

Incorporation of ^{14}C-leucine into protein after aeration of anaerobic, lipid-depleted KD115 cells for various periods: the effects of unsaturated fatty acid (UFA) and chloramphenicol. Cells were aerated in synthetic medium for 2, 7, or 10 hr as indicated, then their ability to incorporate ^{14}C-leucine tested. Tween 80 was added to give a concentration of 5 ml/l (circles); the addition of Tween 80 and chloramphenicol (4 mg/ml) together is indicated by the triangles, while no addition to the basic aeration medium is indicated by the squares.

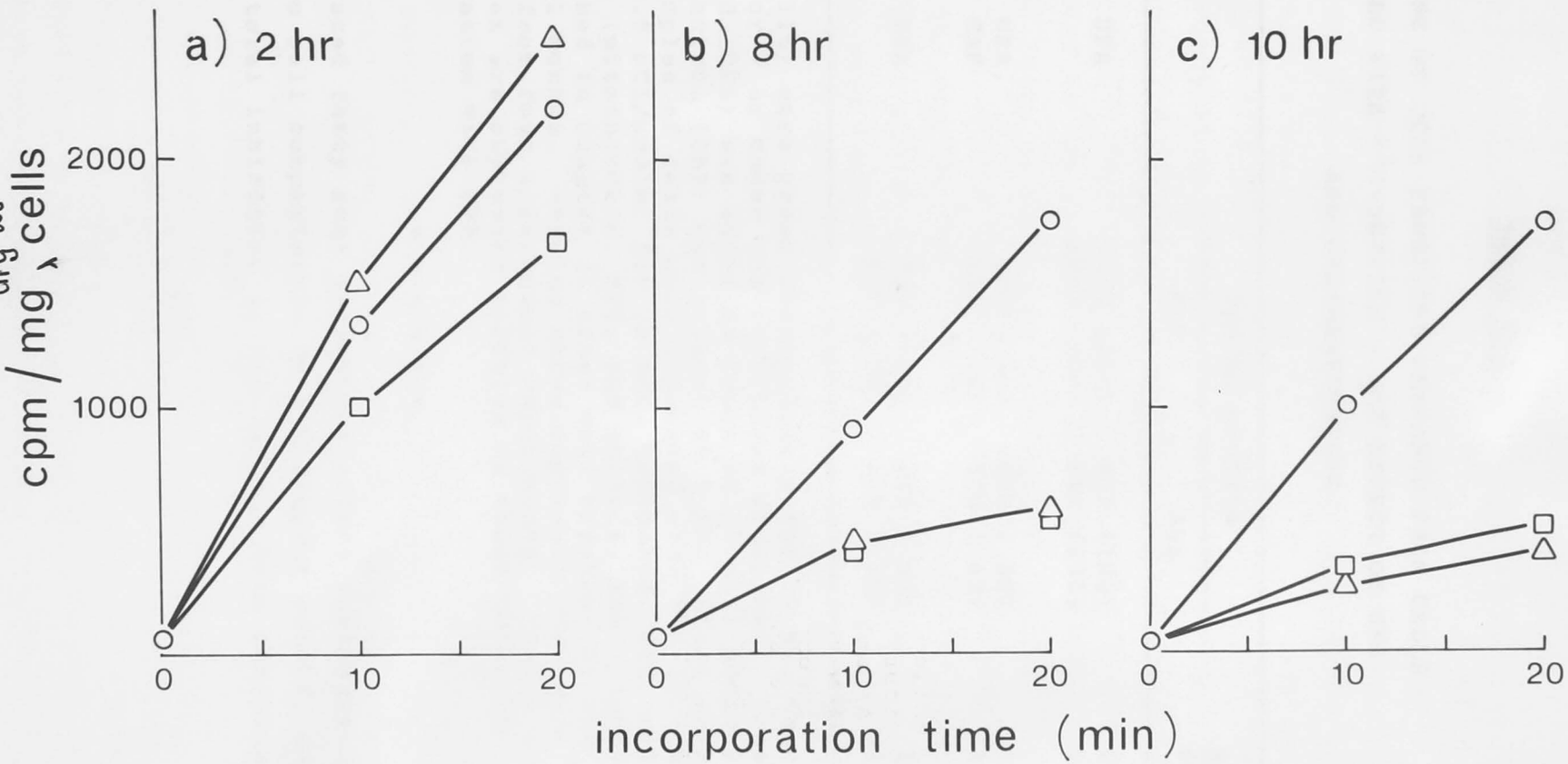

a) 2 hr
b) 8 hr
c) 10 hr
cpm / mg dry wt cells
2000
1000
0
10
20
incorporation time (min)

TABLE 6-5

LABELLING OF CELL FRACTIONS PREPARED FROM CELLS INCUBATED WITH ^{14}C-LEUCINE : THE EFFECT OF UFA AND CHLORAMPHENICOL

	Cpm/mg protein		MIT / SOL
	MIT	SOL	
AERATED, + UFA	1760 (100)	850 (100)	2.07
	1340 (100)	890 (100)	1.51
AERATED, + UFA, + CAP	520 (30)	480 (56)	1.08
	420 (31)	370 (42)	1.13
AERATED, - UFA	730 (41)	450 (53)	1.62
	450 (34)	270 (30)	1.67

Cells (KD115) were grown anaerobically for 22 hr, then aerated for 7 hr under the conditions described. Unsaturated fatty acid (UFA) was added as Tween 80 (5 ml/l medium); chloramphenicol (CAP) was present at 9 mM. After aeration 500 mg samples of cells were incubated with ^{14}C-leucine (3 µCi, 0.5 µCi/µmole) for 20 min, harvested, and cell fractions (mitochondria, MIT, and soluble, SOL) prepared as described in Chapter II after cell breakage by the mechanical method. Results shown represent the range obtained from four independent experiments. Values in parentheses are expressed relative to those obtained after aeration with UFA.

* * * * * *

and unsaturated fatty acid starvation affect incorporation into the two cell compartments to a different extent, even though the total inhibition is similar for both inhibitory treatments.

5. OXYGEN-INDUCED FORMATION OF LIPIDS AND ENZYMES IN THE PETITE MUTANT

The initial approach to the problem using this mutant was to examine the oxygen-induced synthesis of the lipids and mitochondrial enzymes that were discussed in the previous chapter for the wild-type organism. When the petite was grown anaerobically on a lipid-poor medium it reached a state of lipid depletion the same as that found in the wild-type cell (Table 6-6). Aeration of the lipid-deficient petite cells resulted in increases in the amounts of lipids (unsaturated fatty acids, ergosterol, phospholipid) that again were very similar to those of the wild-type cells. However, in contrast to the wild-type, there was no significant induction of any of the mitochondrial enzymes measured when petite cells were aerated. The enzymes measured were fumarase, malate dehydrogenase, cytochrome oxidase, and succinate dehydrogenase. The increase in total phospholipid was relatively small. Also, ubiquinone synthesis during aeration of the petite was very low, but this can be attributed to catabolite repression. Cytochrome c, which is also very sensitive to catabolite repression, was formed during aeration of the petite but in much smaller amounts than those found in chemostat cultures grown under glucose-limiting (derepressed) conditions.

These results raised the possibility that the lack of enzyme synthesis during aeration of anaerobically-grown batch cultures of petite cells was due to severe catabolite repression. However, when the petite was grown in aerobic derepressed (chemostat) cultures there was still no significant synthesis of mitochondrial enzymes. It is interesting to note as a corollary of the above results that the synthesis of unsaturated fatty acids and ergosterol, unlike ubiquinone and mitochondrial enzyme syntheses, are not subject to catabolite repression, (cf. Lukins *et al.* 1968).

6. THE EFFECTS OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON THE INDUCED SYNTHESIS OF LIPIDS : WILD-TYPE VS PETITE

Figure 6-5 shows the time course of synthesis of unsaturated fatty acids and ergosterol in the petite and wild-type strains when lipid-deficient anaerobically-grown cells were aerated. Clearly, in the absence of antibiotics the rates of synthesis of these lipids were the same in the two cell types. In addition, the composition of the fatty acids from both cells before and after aeration were very similar.

As indicated previously, the addition of either chloramphenicol or cycloheximide causes partial inhibitions, in wild-type cells, of both unsaturated fatty and ergosterol syntheses (Fig. 6-5a, c). In contrast, these antibiotics did not inhibit induced lipid syntheses in the petite mutant (Fig. 6-5b, d).

It is important to establish that the lack of inhibition by the antibiotics when the petite is aerated is not due to impermeability. Figure 6-6(a,b) shows the uptake of ^{14}C-chloramphenicol by both the petite and wild-type. In this experiment the cells were incubated with different concentrations of chloramphenicol (at constant specific activity). The cells were then washed three times and assayed for chloramphenicol by determination of radioactivity. After three washes the radioactivity in the supernatant accounted for less than 5 per cent of the cell radioactivity. For both strains the uptake, over two concentration ranges of chloramphenicol, was dependent on the external concentration of chloramphenicol in the same way. Cycloheximide was shown to be active in the petite by its effect on growth : one µg/ml was sufficient to completely stop growth. Also, cytochrome c synthesis on aeration of the petite was inhibited almost completely by the presence of cycloheximide.

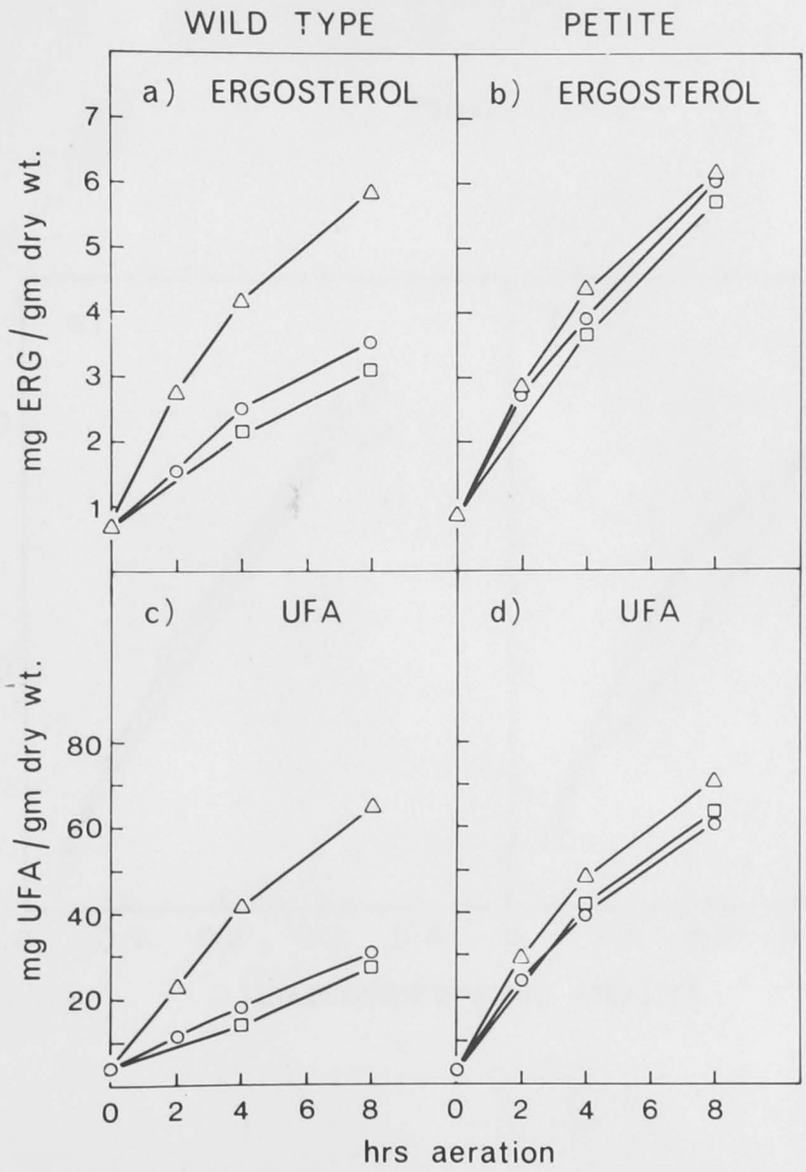
TABLE 6-6

OXYGEN-INDUCED FORMATION OF LIPIDS IN THE PETITE MUTANT

Growth conditions (strain)	Total fatty acid	Unsaturated fatty acid	Ergosterol	Phospholipid	Ubiquinone
Anaerobic (petite or wild-type)	22	3.6	0.5	52	0.01
Aerated 8 hr (petite)	88	71	6.2	66	0.05
Aerated 8 hr (wild-type)	85	70	5.8	68	0.18

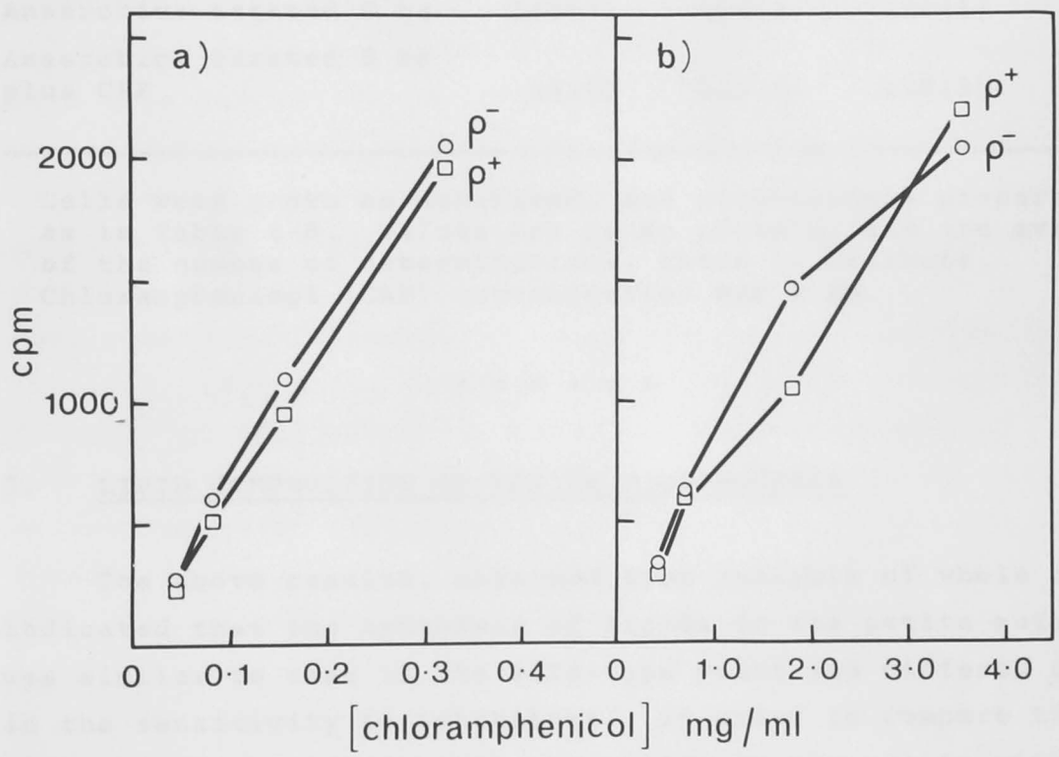
Cells, petite or wild-type, were grown anaerobically for 20-22 hr without lipid supplements, and then aerated as described in Chapter II. Values given are mg/gm dry wt cells, and are taken from typical experiments.

Figure 6-5



Time course of lipid synthesis during aeration of anaerobic, lipid-depleted cells : a comparison of the wild-type and petite strains. The addition of chloramphenicol (4 mg/ml), and cycloheximide (10 µg/ml), to the aeration medium is indicated by the circles and the squares, respectively. Cells aerated without antibiotic addition are indicated by the triangles.

Figure 6-6



The uptake of ^{14}C-chloramphenicol by petite and wild-type cells. The uptake over two concentration ranges is shown; the experimental details are described in the text. Cells were incubated with the label for 20 min at 30°C.

TABLE 6-7

LIPID CONTENT OF WILD-TYPE AND PETITE MITOCHONDRIA

Growth Conditions	Phospholipid		Unsaturated Fatty Acid	
	wild-type	petite	wild-type	petite
Aerobic	272(3)	215(2)	170(3)	185(2)
Anaerobic; aerated 8 hr	143(3)	166(2)	194(3)	212(2)
Anaerobic; aerated 8 hr plus CAP	54(3)	143(2)	116(3)	216(2)

Cells were grown as described, and mitochondria prepared as in Table 6-8. Values are µg/mg protein, and are averages of the number of determinations, shown in brackets. Chloramphenicol (CAP) concentration was 9 mM.

* * * * * *

7. LIPID COMPOSITION OF PETITE MITOCHONDRIA

The above results, obtained from analysis of whole cells, indicated that the synthesis of lipids in the petite mutant was similar to that in the wild-type yeast and differed only in the sensitivity to inhibitors. In order to compare the assembly of the lipids into organelles in the petite with that of the wild-type, we have examined the lipid composition of mitochondria from both strains after aeration of lipid-depleted anaerobic cells. The term 'mitochondria' or 'mitochondrial fraction' is used in the case of the petite to indicate that fraction that is operationally defined as 'mitochondria' in wild-type cells. In wild-type cells this fraction is further defined functionally by respiratory activity, but this is not possible in case of petite cells.

The results of the lipid analysis of mitochondrial fractions from aerated cells are presented in Table 6-7; the lipid composition of mitochondrial fractions of

aerobically-grown cells is included for comparison. It can be seen that mitochondria prepared from either the petite or the wild-type have a very similar lipid composition. Moreover, while the presence of chloramphenicol during aeration resulted in lowered lipid levels in the mitochondria from wild-type cells, this effect is not seen in the petite cells. Hence, in contrast to the wild-type cell, chloramphenicol affects neither the synthesis of the lipids examined nor the incorporation of these lipids into the mitochondrial fraction in the petite cell.

The phospholipid classes of mitochondria prepared from wild-type and petite cells are shown in Table 6-8. A corresponding analysis for mitochondria from *Candida utilis* is presented for comparison to show the differences that can exist between species. The relative amounts of phosphatidyl choline, phosphatidyl inositol, and phosphatidyl serine from petite mitochondria all appeared to be significantly different from those found in the wild-type mitochondria. There is no indication as yet of the physiological significance of these differences, although they may of course represent variations in the response to culture conditions.

TABLE 6-8

PHOSPHOLIPID COMPOSITION OF MITOCHONDRIA FROM WILD-TYPE AND PETITE YEAST

Organism	Distribution (%,w/w)				
	PE	PC	PI	PS	other
S. cerevisiae (ρ^+)	45.3	36.7	1.1	12.5	2.4 (5)
S. cerevisiae (ρ^-)	51.2	22.5	18.4	5.7	0.2 (2)
Candida utilis	18.5	42.9	4.5	27.6	6.5 (3)

Cells were grown aerobically with 2.5% glucose or galactose as carbon source. Mitochondrial fractions were prepared after cell breakage by the mechanical method: the mitochondria were washed twice then pelleted through 40% sucrose, or banded in a sucrose density gradient. Values are given as the average of the number of determinations (in parentheses). Phospholipid classes examined were: phosphatidyl ethanolamine, PE; phosphatidyl choline, PC; phosphatidyl inositol, PI; and phosphatidyl serine, PS.

DISCUSSION

1. THE EFFECTS OF UNSATURATED FATTY ACID RESTRICTION ON THE SYNTHESIS OF MITOCHONDRIAL COMPONENTS IN KD115

The studies presented above, in which the unsaturated fatty acid auxotroph KD115 was used, have attempted to examine the general problem of lipid synthesis - protein synthesis interregulation by following the effects of lipid restriction on various biosynthetic events which involve protein synthesis. It was found that the starvation of the mutant for unsaturated fatty acids had widespread effects on the biosynthetic activities examined.

When KD115 was grown anaerobically on a lipid-poor medium, a lipid depletion similar to that found in the wild-type or petite organisms was obtained, the degree of depletion depending on the extent of growth. The development of respiration on aeration of these lipid-depleted KD115 cells depended both on the extent of lipid depletion that occurred anaerobically and the availability of lipids during the aeration period. Generally, the more unsaturated fatty acid available to the cell, whether endogenous or exogenous, the greater was the induction of respiration. This inhibition of the development of respiration could be correlated with a concomitant inhibition of cytochrome and respiratory enzyme synthesis. These *in vivo* results strongly suggest that lipids are required in the biosynthesis of individual protein components of functional mitochondria, whether strongly membrane-bound (cytochrome oxidase, succinate dehydrogenase) or loosely associated as in the case of the soluble enzymes (fumarase, malate dehydrogenase).

These results are in certain respects at variance with those presented by Proudlock, Haslam, and Linnane (1969) who found that depletion of unsaturated fatty acid in KD115 led only to an inhibition of the synthesis of factors which couple phosphorylation to oxidation, and not to an

inhibition of the development of respiratory capacity. The reason(s) for this conflict are not certain, although aerobic growth/respiration studies (not presented here) in which KD115 was grown aerobically with various levels of unsaturated fatty acid in the medium, suggest that a level of unsaturated fatty acid depletion can be found that leads only to this very selective deletion of synthesis reported by Proudlock *et al.* (1969).

2. THE SPECIFICITY OF THE EFFECTS OF UNSATURATED FATTY ACID STARVATION IN KD115

(a) Effects on catalase synthesis

The inhibition of the development of catalase activity during aeration of KD115 without lipid showed that a non-mitochondrial enzyme was also affected by unsaturated fatty acid starvation. But this may not be true of all non-mitochondrial enzymes. The pathway for the synthesis of the prosthetic group of catalase, heme B, involves at least one mitochondrial enzyme. This is the enzyme protoheme ferrolyase, which combines ferrous iron with protoporphyrin (Lacelles, 1964). However petite cells can synthesise both catalase (Chantrenne, 1954; Chantrenne and Courtois, 1954; Lowdon, unpub. obs.) and cytochrome c, which require heme B and heme C, respectively. These results therefore suggest that the synthesis or activity of the enzymes of the heme pathway is not dependent on mitochondrial protein synthesis or mitochondrial respiration, as both these systems are lacking in petite cells. On the other hand protoheme ferrolyase requires specific lipids for activity (Mazanowska, Neuberger, and Tait, 1966; Yoneyama, Sawada, Takeshita, and Sugita, 1969), so it would seem that the use of catalase as a control enzyme in the type of experiments shown above is limited. Unfortunately there are no other oxygen-inducible

cytoplasmic enzymes known in yeast, with the possible exception of the glyoxylate cycle enzymes.

(b) Effects on Whole Cell Protein Synthesis

The studies on the whole cell incorporation of ^{14}C-leucine into protein provide a better insight into the overall effects of unsaturated fatty acid starvation. These experiments showed that the absence of unsaturated fatty acids during aeration of depleted cells, or the presence of chloramphenicol, led to the same degree of inhibition of incorporation, and suggested that the lipid starvation caused a selective inhibition of the synthesis of mitochondrial proteins.

However when this suggestion was examined in more detail, by fractionation of the cells after labelling under the above conditions, it was found that chloramphenicol inhibition and inhibition by unsaturated fatty acid restriction affected the different cell compartments (mitochondrial and soluble), to a different extent. The distinction between chloramphenicol inhibition and unsaturated fatty acid starvation is also seen on cytochrome c synthesis. As has been seen previously, chloramphenicol inhibited all but the c-type cytochromes and this is consistent with the view that cytochrome c is synthesised on cytoplasmic ribosomes (Kadenbach, 1967; Gonzalez - Cadavid, Bravo, and Campbell 1968), while the synthesis of the particulate cytochromes requires the co-ordinated activity of the mitochondrial and cytoplasmic systems (for example, Kellerman, Griffiths, Hansby, Lamb, and Linnane, 1971). In the absence of added unsaturated fatty acid, however, there is little synthesis of cytochrome c during the aeration of the lipid-depleted KD115 cells. Both the effect on cytochrome synthesis and the incorporation studies suggest that the cytoplasmic and mitochondrial protein-synthesising systems are affected by lipid depletion under the conditions described above. Consequently, the effect of unsaturated fatty acid restriction

on the development of the respiratory apparatus cannot be attributed to a specific mitochondrial inhibition.

3. THE SYNTHESIS OF MITOCHONDRIAL ENZYMES

Several laboratories have stressed that the synthesis of many mitochondrial proteins requires the co-ordinated activity of both the mitochondrial and the cytoplasmic protein-synthesising systems (for references and discussion see Chapter I and introduction to Chapter V). As outlined previously, this concept has been derived largely from a comparison of the *in vivo* and *in vitro* effects of chloramphenicol and cycloheximide. For example, Kellerman *et al.* (1971) in a study of the effects of chloramphenicol and cycloheximide on the incorporation of ^{14}C-leucine into the protein of mitochondrial and soluble fractions of aerobic, batch-grown yeast, estimated that the mitochondrial protein-synthesising system of derepressed yeast synthesised 13 per cent of the mitochondrial protein as this was the degree of chloramphenicol inhibition (cf. estimates discussed in Chapter IV). Under these conditions there was practically no chloramphenicol inhibition of the incorporation into soluble protein while cycloheximide, in contrast, inhibited incorporation into both cell compartments.

The degree of inhibition (approx. 60 per cent) by chloramphenicol during the aeration of KD115 cells, described earlier, was therefore rather surprising, although the same was found to be true during the aeration of lipid-depleted wild-type cells. Presumably this is a consequence of the aeration conditions which are designed to favour rapid mitochondrial development. In the present study chloramphenicol, even though it preferentially inhibited the incorporation into mitochondrial protein, also caused a large inhibition of the incorporation into soluble protein. This suggests that a large portion of the inhibition observed during aeration in the presence of chloramphenicol

is due to inhibition of protein synthesis on the cytoplasmic ribosomes. Hence, no estimate of the proportion of mitochondrial protein synthesised by the mitochondrial protein-synthesising system can be made by this method under these conditions.

The inhibition of the synthesis of mitochondrial enzymes by chloramphenicol and erythromycin, as well as cycloheximide, during the aeration of lipid-depleted wild-type cells can be seen as an indication of this interdependence between the two protein-synthesising systems. As outlined in the discussion of Chapter V, it is proposed that product(s) of the mitochondrial protein-synthesising system are necessary for the synthesis of enzymes such as fumarase, succinate dehydrogenase, and malate dehydrogenase, even though these syntheses occur on cytoplasmic ribosomes.

The hypothesis presented above is supported by the results obtained with the petite mutant. This mutant lacks mitochondrial protein-synthetic activity (Wintersberger, 1967; Kuzela and Grecna, 1969; Schatz and Saltzgaber 1969a; Kellerman *et al.* 1971). Therefore the synthesis of enzymes which require the activity of this system should be inhibited. The lack of respiratory enzyme or membrane-bound cytochrome synthesis in the petite is consistent with their postulated requirement for product(s) of the mitochondrial protein-synthesising system.

However, a refinement of this concept is necessary to explain the effects of protein synthesis inhibitors and the petite mutation on lipid synthesis.

4. LIPID SYNTHESIS IN THE PETITE MUTANT : THE COUPLING OF LIPID AND PROTEIN SYNTHESES

In contrast to the extreme effect of the petite mutation on the synthesis of respiratory enzymes, this mutation has little effect on the synthesis of lipids. The rate of synthesis of ergosterol and unsaturated fatty acids during

aeration of lipid-depleted petite cells was the same as that seen in wild-type cells cultured and aerated in the same way. Likewise, Kovac, Subik, Russ, and Kollar (1967) have reported that aerobically-grown wild-type and petite cells contain the same lipid levels. However the results presented show that induced lipid synthesis in the petite is not sensitive to chloramphenicol or cycloheximide, even though the concentrations of these antibiotics used inhibited induced lipid synthesis in wild-type cells by about 60 per cent.

Both the lack of effect of the *L-isomer* of chloramphenicol on the synthesis of lipids and enzymes in the wild-type cell (described in the previous chapter), and the lack of effect of the *D-isomer* in the petite where there is no mitochondrial protein synthesising system, indicate that the inhibitions of these syntheses in the wild-type cells by the *D-isomer* is a consequence of an inhibition of a functional mitochondrial protein-synthesising system.

It is apparent that in the petite the syntheses of lipids such as unsaturated fatty acid, ergosterol and phospholipid, as well as ubiquinone, can occur quite independently of the activity of a mitochondrial protein-synthesising system, or the synthesis of mitochondrial enzymes. Also, the mitochondria from petite and wild-type cells have a similar lipid composition. These observations indicate that the synthesis of lipids and their assembly into a mitochondrial fraction can proceed independent of the synthesis of inducible components of some respiratory enzymes. Other mitochondrial proteins, such as ATPase (F_1) and cytochrome c, both of which are synthesised on the cytoplasmic ribosomes, appear to be under a different control mechanism as they are synthesised in the petite.

In wild-type cells, where under most conditions the mitochondrial protein-synthesising system is present, the situation is different; the inhibitor studies shown in this and the previous chapter revealed that lipid synthesis

was partially coupled to protein synthesis during aeration of lipid-depleted anaerobes. In the case of the synthesis of mitochondrial enzymes, coupling results from the necessity for products from both systems before respiratory enzymes are formed. However the nature of the coupling between lipid synthesis and protein synthesis associated with mitochondrial development must be characteristically different, because, as shown by the petite study, lipid synthesis is not dependent on the concurrent formation of products of the mitochondrial protein-synthesising system.

Another important distinction has been revealed by the lack of effect of cycloheximide on induced lipid synthesis in the petite. (In the wild-type cycloheximide caused a partial inhibition during aeration in both lipid-supplemented and lipid-depleted cells). The results obtained with the wild-type cells were difficult to interpret, because of the partial effects seen, but the results obtained with petite cells provide a means of rationalization. The fact that at least partial syntheses of unsaturated fatty acid, ergosterol, and ubiquinone could occur in the presence of cycloheximide in the wild-type cells suggested that the enzyme systems for these syntheses could be formed under anaerobic conditions, even though there was no actual synthesis of these compounds. The partial inhibition of the synthesis of these lipids in the wild-type cells could be viewed as an inhibition of new enzyme synthesis, and a decay of pre-existing amounts of the enzymes, with a resultant decrease in lipid formed. Alternatively, it could be viewed as an inhibition which is the consequence of the coupling of mitochondrial and cytoplasmic systems with lipid synthesis. The lack of cycloheximide inhibition in the petite makes an important distinction between these possibilities : the former is untenable and the latter postulate of interdependence between the systems is supported.

This leaves the interesting situation where lipid synthesis during aeration of the petite is not dependent on

the products of either protein synthesising system, nor is the assembly of these lipids into the organelle dependent on any protein synthesis under these conditions. It appears that a consequence of the loss of the mitochondrial protein-synthesising system in the petite is a loss of the dependence of lipid synthesis during aeration on either protein-synthesising system : the coupling that existing in wild-type cells between lipid synthesis and protein synthesis has been dissociated. But, as described above, this coupling mechanism must be different from that which associates the two protein-synthesising systems of the cell during the synthesis of respiratory enzymes.

5. THE SYNTHESIS OF ERGOSTEROL IN WILD-TYPE AND PETITE YEAST

The results presented by Parks and co-workers (Starr and Parks, 1962; Parks and Starr, 1963; Adams and Parks, 1969) on the control of ergosterol synthesis are worth special comment, as the conclusions these workers reached conflict in some ways with those presented above. These investigators reported that conditions which induce petite formation (acriflavin, heavy metal ions, high temperature) also inhibited sterol formation, and concluded that there was a close correlation between suppression of sterol synthesis and loss of respiratory capacity (Parks and Starr, 1963). In contrast, the results presented in this chapter show that sterol synthesis can proceed normally in a petite mutant, independent of respiratory function.

Later, Adams and Parks (1969) reported an inhibition of ergosterol synthesis by chloramphenicol during aeration, and interpreted this to mean that a component of sterol formation was synthesised by the mitochondrial protein-synthesising system. They suggested that this component may be "at least some of the enzymes or some regulatory component involved in sterol synthesis", the implication being that the

regulatory component was necessary before sterol synthesis could take place. In contrast, our studies on ergosterol synthesis in the petite mutant, where there is no functional mitochondrial protein-synthesising system, show that the rate of ergosterol synthesis is the same as that in the wild-type. This indicates that the nature of the control that the mitochondrial protein-synthesising system exerts on ergosterol (and unsaturated fatty acid) synthesis is much more subtle, and that the regulatory component is not obligatory for synthesis.

CHAPTER VII

CONCLUDING REMARKS

The results presented have been discussed in the context of other studies, past and concurrent, of the biogenesis of mitochondria. In part, the experiments have been designed to complement studies on the biogenesis of mitochondrial proteins, particularly those of Vary *et al.*(1969, 1970). For this reason attention has been concentrated on lipid synthesis and the effects of lipid synthesis on developmental events associated with the formation of fully functional organelles. Although the results have been discussed in the individual chapters, it seems worthwhile to attempt to present an overall view.

1. YEAST PHYSIOLOGY : THE IMPORTANCE OF LIPIDS

(a) Anaerobic growth and cellular lipid content

S. cerevisiae is a facultative anaerobe only while growth remains unrestricted by exogenous unsaturated fatty acids and sterols. After anaerobic growth on unsupplemented media the yeast fatty acids are reduced in amount and are predominantly saturated, the phospholipid content is reduced by about half, the ergosterol content is markedly depleted, and ubiquinone appears to be absent. Cardiolipin, a specific mitochondrial phospholipid, is also reduced in amount (Jakovcic *et al.* 1971).

These analyses make an interesting comparison with others carried out on *Mucor genevensis* in collaboration with Dr. G.D. Clark-Walker, and presented elsewhere (Gordon, Stewart, and Clark-Walker, 1971). *M. Genevensis* is also a facultative anaerobe, and shows a dimorphism (yeast-mycelial) which is dependent on growth conditions. In an investigation

of the relationship between this dimorphism and the lipid content (Gordon *et al.* 1971),we found that aerobic growth and anaerobic growth of *M. genevensis* affect the cellular levels of unsaturated fatty acids and ergosterol in the same way as that described for *S. cerevisiae*. Thus after aerobic growth the fatty acids of *M. genevensis* cells were predominantly unsaturated (approximately 80 per cent) and the content of ergosterol was likewise high. After anaerobic growth the fatty acids present were mainly saturated, and the ergosterol content was low. However, there appeared to be no correlation between the morphology of *M. genevensis* and its lipid content. It is interesting to note that supplementation of the anaerobic growth medium with ergosterol and Tween 80, or even the complete lipid extract of aerobic mycelium, did not result in prolonged anaerobic growth of *M. genevensis;* nor were exogenous lipids incorporated into the cells. This is in contrast to the effects of lipid addition on the anaerobic growth of *S. cerevisiae*.

(b) Effect of lipids on biosynthetic activity

As was discussed in Chapter IV, the anaerobic growth conditions can have quite profound effects on biosynthetic activities of *S. cerevisiae*. These effects appear to be related partly to the lack of lipids and partly to the lack of oxygen, and some distinction between these can be made by a comparison of lipid-depleted and lipid-supplemented anaerobic cells.

Anaerobic propagation of *S. cerevisiae*, with or without added lipids, does not result in the deletion of the mitochondrial structure or the mitochondrial genome (see Chapter I). However the expression of the genome, and organelle composition, can be radically affected by the absence of oxygen. Promitochondria from lipid-supplemented cells have very low levels of the aerobic cytochromes, particulate respiratory enzymes, and ubiquinone; consequently

respiration is very low. However other mitochondrial functions are still operative, although at a reduced level. Schatz and co-workers (see earlier) have found that at least some energy-coupling activities are present, and Vary *et al.* (1970) and Rouslin and Schatz (1969) have proposed that product(s) necessary for mitochondrial enzyme synthesis are accumulated in supplemented cells. All these functions require the activity of the (pro)mitochondrial protein-synthesising system, and the presence of such a system has been directly demonstrated (Chapter IV). It appears that in the absence of oxygen, but with lipids, the mitochondrial protein-synthesising system remains active and is <u>partially</u> expressed, i.e. oxygen does not directly control the formation of the mitochondrial protein-synthesising system in this type of cell, although the <u>full</u> expression of this system presumably does require oxygen.

It is clear that lipid depletion results in effects additional to those caused by anaerobiosis. The lipid composition of promitochondria from depleted cells is severely altered. Probably the most important consequence of this lipid stress is the loss of mitochondrial and cytoplasmic protein synthetic activity and RNA synthetic activity. There also appears to be a specific loss of mitochondrial RNA, the possible importance of which is discussed below.

(c) <u>The role of lipids in maintaining the activity of the mitochondrial protein-synthesising system</u>

In discussing the possible reasons for the loss of activity of the mitochondrial protein-synthesising system (MPSS) it is helpful to consider at the same time the experiments relating to the re-establishment of this system. The possibility that lipids are required only for the 'structural organisation' of the MPSS can be discounted as the re-establishment of this system requires new protein synthesis and most likely new mitochondrial RNA synthesis.

Experiments showing that mitochondrial RNA is not present in lipid-depleted isolated promitochondria (Forrester *et al.* 1971a, b; Chapter IV) need careful interpretation, partly because this is a negative result and is subject to experimental difficulties. For example, the changed membrane composition may lead to loss of mitoribosomes during isolation of promitochondria. However several proposals can be advanced. It is possible that mitoribosome binding to membranes and mitochondrial ribosomal RNA synthesis is dependent on the lipid composition of the mitochondrial membrane, so that a changed membrane composition restricts the synthesis of mitochondrial RNA (Forrester *et al.* 1971a, b). However, one also has to account for the preferential loss of mitochondrial RNA, and explain why the cytoplasmic RNA content appears unaffected.

The evidence presented in Chapter IV shows that anaerobic growth without lipids results in a general loss of protein and RNA synthetic capacity in the cell. We have suggested a modification of the hypothesis presented above in which mitochondrial RNA turns over more rapidly than cytoplasmic RNA. (Suggestive evidence for this more rapid turnover is presented in Chapter IV). If this is so then a general shut-down of RNA synthesis would result in the selective loss of mitochondrial RNA, over the relatively short time that RNA synthesis is inactive, and it is not necessary to postulate that membrane structure selectively controls the synthesis of mitochondrial RNA.

However the situation may be more complex. The fact that cycloheximide inhibits the formation of the MPSS during aeration of lipid-depleted anaerobes (Chapter IV) shows that the synthesis of proteins is also required for the re-establishment of this system. The loss of mitochondrial RNA during anaerobic growth may be but partial explanation of the loss of mitochondrial protein synthesis, as it is possible that loss of ribosomal proteins, for example, is also involved.

2. THE MANY FACETS OF MITOCHONDRIAL ENZYME SYNTHESIS

The use of antibiotics such as erythromycin and chloramphenicol that selectively inhibit mitochondrial protein synthesis, together with the use of different anaerobic culture conditions and different strains, has revealed that classes of mitochondrial enzymes are subject to very different control mechanisms. These experiments have been described by Vary *et al.* (1969, 1970) and Vary (1970), and are summarized in Chapters V and VI.

The synthesis of the cytochrome oxidase complex can be readily differentiated from other mitochondrial enzymes by its low threshold of sensitivity to chloramphenicol and erythromycin, and by its sensitivity to these drugs during aeration of lipid-supplemented anaerobes. Results such as these have been interpreted to mean that the formation of cytochrome oxidase requires oxygen-induced products from both the cytoplasmic and mitochondrial protein synthesising system (Henson *et al.* 1968b; Chen and Charalampous, 1969; Schiefer, 1969; Vary *et al.* 1970; Vary, 1970). However the results of Tuppy and Birkmayer (1969) suggest that the product made by the mitochondrial system is cytohemin, and that all the protein components of cytochrome oxidase are synthesised on cytoplasmic ribosomes.

The control of the synthesis of the TCA-cycle enzymes appears to be quite different to that described for cytochrome oxidase. Firstly, both malate dehydrogenase and fumarase are present in considerable amounts after anaerobic growth, and these levels appear to be constitutive as they are not affected by any of the anaerobic growth conditions used (Vary, 1970). In this respect these two TCA-cycle enzymes can be differentiated from succinate dehydrogenase, the amount of which is affected by the anaerobic growth conditions. However in other ways (for example, sensitivity of the oxygen-induced components to antibiotics) the TCA-cycle enzymes are regulated in a similar fashion. It appears that the TCA-cycle enzymes are synthesised on

cytoplasmic ribosomes, and that this synthesis is regulated by a product of the mitochondrial protein-synthesising system.

In part, the synthesis of the ATPase complex is similar to that of the TCA-cycle enzymes. For example, inhibition of the formation of ATPase by chloramphenicol on aeration of anaerobic cells follows the same pattern as that observed for succinate dehydrogenase. But the regulation of ATPase synthesis appears to be more complex. At present, the ATPase system of yeast (Tzagoloff, 1970) and beef heart (Bulos and Racker, 1968) has been resolved into three components (see Chapter V). Evidence has been presented (Chapter V) showing that the F_1 component (soluble ATPase) is synthesised on cytoplasmic ribosomes, and that this synthesis is independent of product(s) of mitochondrial protein synthesis (cf. TCA-cycle enzymes). If this is so then it is difficult to explain why chloramphenicol inhibits ATPase synthesis during aeration of lipid-depleted anaerobes, unless the chloramphenicol inhibition of lipid synthesis under these conditions is involved in some way. The lipoprotein component(s) of ATPase appear to be synthesised by the mitochondrial system, under control of the mitochondrial genome.

The regulation of cytochrome c synthesis represents yet another type of control, possibly the simplest since there appears to be very little dependence on the function of the mitochondrial protein-synthesising system. Thus cytochrome c synthesis continues in petite mutants, and even during aeration of lipid-depleted anaerobes in the presence of chloramphenicol, a condition that inhibits the synthesis of all other mitochondrial proteins examined.

It is clear that the syntheses of these proteins are subject to a complex hierarchy of controls, and that these controls are not necessarily interdependent. Because of this multiplicity of control mechanisms affecting mitochondrial enzyme synthesis it might be expected that

different mitochondrial lipids would also be subject to different controls, but this is not generally true.

3. ANTIBIOTICS AND INDUCED LIPID SYNTHESIS

The evidence presented (Chapters V and VI) shows that the effects of the inhibitors of protein synthesis on oxygen-induced lipid synthesis differ in at least two important aspects from those described above on enzyme synthesis:

(i) only partial inhibitions are observed in the wild-type organism (the sensitivity for inhibition of lipid synthesis by these antibiotics is lower than that for inhibition of enzyme synthesis);

(ii) the petite mutation has no direct effects on lipid synthesis, and indeed the partial sensitivity to inhibitors detected in the wild-type is not observed in the petite mutant.

Given that the partial inhibitions of lipid syntheses cannot be attributed to secondary effects (see Chapters V and VI) it is concluded that these inhibitions are in some way a consequence of the inhibition of protein synthesis in the organelle. We have suggested that the inhibition of lipid synthesis is a result of an interregulatory mechanism that couples lipid and protein synthesis, although it is evident that this coupling is not tight.

In the petite cell coupling of mitochondrial protein synthesis and lipid synthesis is apparently lost : the effect on lipid synthesis of the mutation to the petite (and the loss of the mitochondrial protein-synthesising system) is a loss of sensitivity to inhibitors of mitochondrial and cytoplasmic protein synthesis. This loss of interdependence is emphasised by the fact that TCA-cycle enzymes or cytochrome oxidase are not synthesised in the

petite, and yet lipid synthesis is near normal. It is probable that the lack of interregulation in the petite is a consequence of the genetic deletion of the mitochondrial protein-synthesising system, i.e. the interregulation depends upon some product of the mitochondrial system. However, lipid synthesis during aeration of petite cells is also insensitive to cycloheximide. This observation suggests that mitochondrial products are involved in the regulation of whole cell membrane synthesis (cf. Watson and Lowenstein, 1970). Supporting this postulate, we have found that chloramphenicol effects on lipid syntheses (unsaturated fatty acids, phospholipids) are spread over whole cell membrane, even though this antibiotic appears to specifically inhibit mitochondrial protein synthesis. An apparent exception was ergosterol synthesis during aeration of lipid-depleted cells : in the presence of chloramphenicol inhibition seen at the intact cell level was not seen in derived mitochondrial fractions. Another possible difference was revealed during the examination of ubiquinone synthesis, as indicated below.

Because ubiquinone is absent from both anaerobic cell types (in contrast to ergosterol and unsaturated fatty acids) it is possible to compare its synthesis and incorporation into mitochondria on aeration. The studies presented in Chapters III and V demonstrated that ubiquinone synthesis was subject to the same environmental control (catabolite repression, oxygen tension) as other respiratory-chain components. In this respect ubiquinone levels reflect the degree of mitochondrial development. So far, ubiquinone is unique as a non-protein marker for mitochondrial development in cells. Nevertheless conditions could be defined where ubiquinone synthesis proceeded independent of the synthesis of other elements of the electron-transfer chain.

The effect of chloramphenicol and cycloheximide on ubiquinone formation during aeration of both anaerobic cell types was greater when examined in isolated mitochondria,

compared with intact cells. This may indicate an additional effect of the antibiotics on the assembly of ubiquinone into the respiratory chain. However it is also possible that in the presence of antibiotics the organelle structure formed is more fragile, or that the binding is weaker, so that during isolation there is a loss of components that are normally tightly bound to the organelle.

4. THE ROLE OF LIPIDS IN THE DEVELOPMENT OF THE MITOCHONDRIAL PROTEIN-SYNTHESISING SYSTEM

The question of the role or function of lipids in the development of the mitochondrial protein-synthesising system is basic to the present study, and most likely is closely related to the role of lipids in the maintenance of this system under anaerobic conditions, discussed above. Results presented in Chapter IV showed that conditions could be defined where lipid-depleted anaerobic cells were induced to re-establish mitochondrial protein-synthetic activity independent of further lipid synthesis. These results pose a certain dilemma. On one hand lipid depletion under anaerobic conditions leads to the loss of mitochondrial protein synthetic activity. On the other, this activity could be re-established without apparent release from this state of depletion. It seems necessary to postulate that lipid depletion under anaerobic conditions has different effects to those obtained aerobically.

In this regard it is relevant to consider the effects of lipid depletion in the unsaturated fatty acid auxotroph, KD115. As was the case with wild-type cells, growth under anaerobic conditions resulted in depletion of lipids and respiratory enzymes. This of course is to be expected since wild-type cells are unsaturated fatty acid auxotrophs in the absence of oxygen. However, when anaerobic lipid depletion of KD115 was not extensive, subsequent mitochondrial development could be induced by oxygen without need for

unsaturated fatty acid in the aeration medium. That is, the endogenous content of these essential lipids was sufficient to support extensive development of mitochondrial function.

This latter observation may provide some insight into the reason why the mitochondrial protein-synthesising system can develop in depleted wild-type cells without further lipid synthesis. Almost certainly, the development of this system does require some lipid, but provided that the overall cellular depletion is not too severe this requirement is not revealed. Presumably, some internal reorganisation of endogenous lipid takes place to enable mitochondrial components, and perhaps other substructures, to be formed. These experiments point to lipids as important regulators of the biosynthetic activities of cells. Almost certainly this role is played by way of their involvement in membrane function.

5. THE FUTURE STUDY OF MITOCHONDRIOGENESIS

Increasingly, the study of mitochondrial biogenesis has come to be viewed as the study of the expression of a small genetic system, and the interaction of this system with nuclear genes. In *S. cerevisiae* the factors that control the expression of mitochondrial DNA, and the nature of the products coded by this DNA, have been reasonably well defined. This definition is well-illustrated by the comparison of lipid-supplemented anaerobic cells, and aerobic cells. As discussed above, the mitochondrial DNA of these cells is transcribed to form mitochondrial ribosomal RNA (at least) and a functional mitochondrial system is present. Under aerobic conditions extra transcripts of the mitochondrial RNA are formed (Fukuhara, 1967b). It appears that oxygen directly controls the expression of part of the mitochondrial genome. Catabolite repression on the other hand in some way restricts this expression.

Of course, the precise mechanisms by which oxygen and fermentable sugars control the expression of mitochondrial DNA are still unknown. At least the nature of the problem is now clearly outlined. In the future it should be possible to show whether or not oxygen directly interacts with mitochondrial DNA.

Another key aspect of a fuller understanding of the biogenesis of this organelle is the way in which products of the mitochondrial and cytoplasmic biosynthetic systems (and the mitochondrial and nuclear genes) interact in the formation of mitochondrial membranes. This thesis has attempted to delineate some of the interactions that do occur, but the detailed mechanisms and the nature of the postulated products have yet to be characterized. If many mitochondrial enzymes and lipids are synthesised on cytoplasmic ribosomes, as appears likely, then problems involving transport and assembly of these components are evident. Again, very little is known about these processes. If mitochondria were originally endosymbionts, one wonders at the evolution of these complex interactions.

The intricacies of membrane structure still remain largely unresolved. However the flexibility of membrane composition, as illustrated by a comparison of the promitochondria from lipid-depleted cells and the mitochondria from aerobic cells, would stress caution in postulating 'a membrane structure for all occasions'. There may in fact be several interconvertible membrane structures which exist in different physiological conditions. More detailed physicochemical studies of mitochondrial membranes appear to be a fruitful approach to such questions.

APPENDIX A

STANDARD METHODS

1. DETERMINATION OF DRY WEIGHT OF YEAST

The cell density of a culture was normally determined by measuring its optical density (O.D.) at 640 nm and converting this to dry weight (w) of cells in mg/ml according to the formula

$$w = x \times O.D. \times \text{dilution factor}$$

where x was a conversion factor determined from a calibration curve. The relationship held over the O.D. range 0.05-0.7 : dry weights are expressed as mg/ml.

2. RNA ESTIMATION

RNA of cell-free fractions was extracted by a modification of the method of Hutchinson and Munro (1961). Protein and nucleic acids were precipitated and washed with cold 7.5 per cent TCA twice, washed twice with absolute ethanol, once with acetone, defatted at 65° for 15 min in ethanol-ether (1:1), and washed in ether. The dried residue (5-10 mg of protein) was resuspended in 1.0 ml of 0.3M potassium hydroxide and hydrolysed for 16 hrs at 37°. The hydrolysate was brought to 1M with respect to perchloric acid and the precipitate removed by centrifugation. The clear supernatant was used for estimation of free nucleotides by the orcinol method of Kerr and Seraidonian (1945) or by UV absorption measurement at 260 nm, using the extinction coefficient, $A^{1\%}_{1cm} = 306$.

3. PROTEIN ESTIMATION

Protein concentration of preparations was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

4. ENZYME ASSAYS

All assays were carried out at 30°C. Spectrophotometric assays were made with the Gilford 2000 Recording Spectrophotometer.

a) Malate dehydrogenase (L-malate: NAD oxidoreductase, E.C.1.1.1.37.) was assayed spectrophotometrically at 340 nm by the disappearance of NADH in the presence of oxaloacetate. The reaction cuvette contained 0.56 µmoles sodium oxaloacetate, 125 µmoles potassium phosphate (pH 7.4), and the enzyme in a final volume of 2.8 ml. Provided enzyme solutions were diluted so that activity did not exceed 0.2 ODU/min strict proportionability between rate and amount of enzyme was obtained. Under these conditions blank rates in the absence of oxaloacetate were not significant. Activity is expressed as µmoles NADH oxidised/min/mg protein.

b) Fumarase (fumarate hydratase E.C.4.2.1.2.) was measured by following the change in absorbance at 240 nm in a reaction mixture containing 135 µmoles potassium phosphate (pH 7.4), 40 µmoles sodium malate, and the enzyme in a total volume of 3.0 ml. Activity is expressed as µmoles fumarate formed/minute/mg protein.

c) Cytochrome oxidase (cytochrome c : o_2 oxidoreductase E.C.1.9.3.1.) was assayed essentially by the method of Smith (1955). 0.4 ml of reduced cytochrome c preparation (180µM) was added to 2.5 ml of 0.05M phosphate

buffer (pH 7.4) and the reaction initiated by adding 0.1 ml of enzyme preparation. The activity was found to be sensitive to cyanide. Activities are expressed as µmoles cytochrome c oxidised/minute/mg protein rather than first order reaction constant as recommended by Smith. Since the initial rate is proportional to the reduced cytochrome c concentration of the reaction mixture activities of a number of preparations expressed in these units were only compared if the assays were carried out on the same day with the same cytochrome c preparation.

d) Succinate dehydrogenase (succinate : paramethosulphonate oxidoreductase E.C.1.3.99.1.). A modified method of Arrigoni and Singer (1962) was used. The reaction mixture containing 50 mM potassium phosphate buffer (pH 7.4), KCN (1.6mM), succinate (16mM) and 0.2 ml of a suitable dilution of enzyme was incubated for 5 minutes before the addition of 0.2 ml phenazine methosulphate (10 mg/ml) and 0.1 ml of dichlorophenolindophenol (0.32 mg/ml) at 30 second intervals (phenazinemethosulphate + dichlorophenolindophenol = paramethosulphonate). The rate in this complete reaction mixture was always assayed simultaneously with the rate of a blank mixture lacking succinate, and this blank rate subtracted. At this concentration of phenazine methosulphate, the reaction velocity was greater than 90 per cent of the maximum velocity as calculated from Lineweaver-Burke plots. Activity was therefore expressed as the initial velocity in µmoles paramethosulphonate reduced/min/mg protein.

e) Catalase (H_2O_2 : H_2O_2 oxidoreductase, E.C.1.11.1.6.). The method involves a colorimetric titration with $KMnO_4$ of substrate (H_2O_2) not decomposed by catalase (Bonnichsen, Chance and Theorell, 1947). 0.2 ml of enzyme preparation was incubated for 15 minutes in 50 ml of 0.024 per cent H_2O_2. The reaction mixture was stopped by the addition of 5 ml concentrated HCl. The solution was

titrated against 0.01M $KMnO_4$ until pink colour just persisted. A blank incubation was also performed by adding HCl at the commencement of incubation. The activity of the enzyme sample is expressed as μmoles H_2O_2 decomposed/minute/mg protein.

5. SUGAR ESTIMATIONS

Galactose concentration was estimated essentially by the method of Scott and Melvin (1953). Glucose was estimated by glucose oxidase reagent (Glucostat Reagent, Worthington Biochemicals, U.S.A.).

6. CELL VIABILITY

Cell viability was measured by the buffered methylene blue staining procedure of Gurr (1965). In some experiments viability was measured by a cell plating procedure.

7. PREPARATION OF ^{14}C(U)-4-HYDROXYBENZOIC ACID

^{14}C(U)-4-hydroxybenzoic acid was prepared from ^{14}C(U)-tyrosine by the alkali fusion method described by Rudney and Parson (1963).

APPENDIX B

STUDIES ON THE DISTRIBUTION OF MITOCHONDRIAL COMPONENTS

Initially it was hoped that it would be possible to follow the distribution of 'mitochondrial' lipids and enzymes between mitochondrial and cytoplasmic cell compartments during the aeration process in which these components are formed. For example, if ubiquinone is synthesised in the cytoplasm then it should be possible to show a transfer from cytoplasm to mitochondria as aeration proceeds. However it has not been possible to carry out completely satisfactory distribution experiments with yeast, for the reasons discussed below.

It was thought that a gentle cell breakage method was necessary, so cell fractions were prepared after lysis of protoplasts formed by snail enzyme digestion of the cell walls. Preliminary experiments gave erratic results, so the method was examined in some detail.

1. EFFECT OF PRE-INCUBATION CONDITIONS ON THE KINETICS OF SNAIL ENZYME DIGESTION

The method of snail enzyme digestion is outlined in Chapter II. Washed cells are resuspended in buffered mercaptoethanol solution; after an incubation period the cells are collected and washed in 0.9M sorbitol buffer, then digested with snail enzyme. The digestion process is followed by optical density measurements. Figure A-1 shows the kinetics of snail enzyme digestion after cells had been subjected to various preincubation conditions, as described in Table A-1 : the variables were mercaptoethanol concentration, pH, and temperature.

TABLE A-1

PREINCUBATION CONDITIONS FOR SNAIL ENZYME DIGESTION

Sample number	Mercaptoethanol[1]	pH	Temperature(°C)
1	0.10	9.3	30
2	0.10	8.0	30
3	0.02	9.3	30
4	0.02	8.0	30
5	0.00	9.3	30
6	0.02	9.3	0

Cells were preincubated for 10 min at the temperature shown then subjected to snail enzyme treatment, the results of which are shown in figure A-1.

1. Values are in molar units.

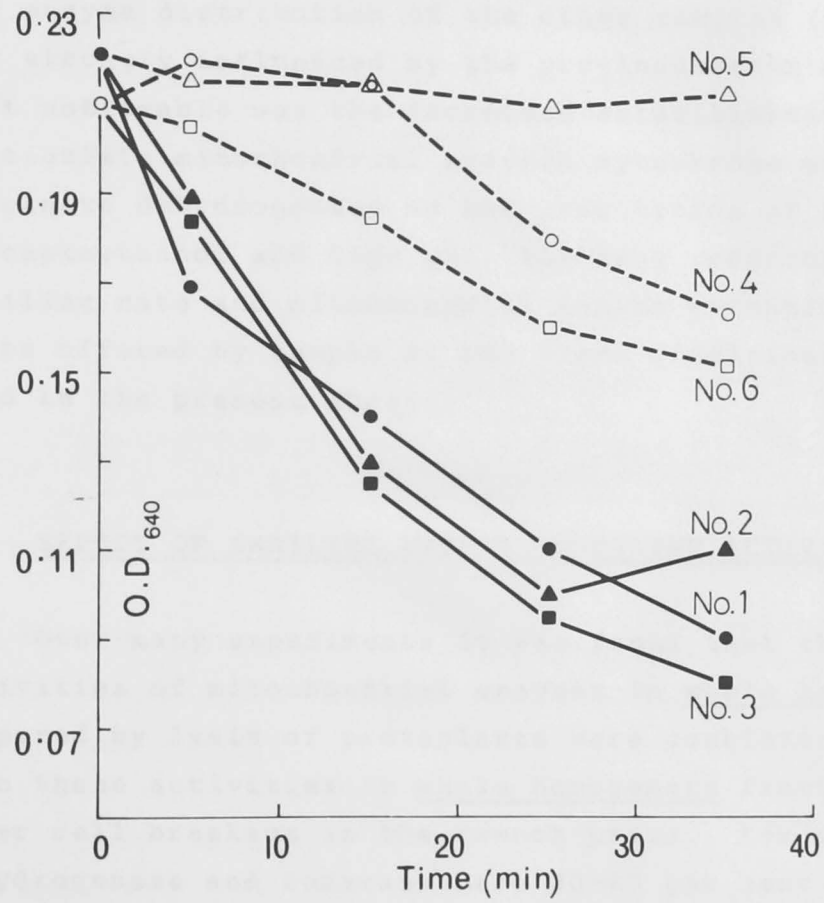
* * * * * * *

The kinetics show the necessity of mercaptoethanol, and the necessity for either high mercaptoethanol concentration (0.1M) or high pH (9.3); if both are low then the digestion is considerably slowed. The efficacy of the preincubation is also temperature dependent. It is important to note that mercaptoethanol is unstable in dilute solution : in the experiments described it was added to the buffer just prior to use, and the pH re-adjusted.

2. EFFECT OF PRE-INCUBATION CONDITIONS ON ENZYME DISTRIBUTION

The preincubation conditions and the extent of snail enzyme digestion of this experiment are summarized in Table A-2(a). After snail enzyme digestion, the protoplasts were washed in the sorbitol buffer twice, then lysed as

Figure A-1



Kinetics of snail enzyme digestion after pre-incubation under various conditions. The digestion is followed by the decrease in optical density at 640 nm. The numbers describing the curves refer to the pre-incubation conditions detailed in Table A-1. Broken lines indicate treatments that severely limited the digestion.

described in Chapter II. Cell debris was removed, and mitochondria were spun from the protoplast-free supernatant (20 min, 20,000g). Mitochondrial and cytoplasmic (post-mitochondrial supernatant) fractions were assayed for the enzymes detailed in Tables A-2(b).

Table A-2(a) shows, like figure A-1, that the low mercaptoethanol, low pH treatment severely restricted the snailing process : this sample was not further analysed. The enzyme distribution of the other samples (Table A-2(b)) was strongly influenced by the pre-incubation conditions. Most noticeable was the increased solubilization of the particulate mitochondrial enzymes cytochrome oxidase and succinate dehydrogenase by the combination of high mercaptoethanol and high pH. The best compromise between snailing rate and mitochondrial enzyme retention appeared to be offered by sample 4, and these conditions have been used in the present thesis.

3. EFFECT OF SNAILING METHOD ON ENZYME ACTIVITY

Over many experiments it was found that the specific activities of mitochondrial enzymes in whole homogenates prepared by lysis of protoplasts were consistently lower than these activities in whole homogenate fractions prepared after cell breakage in the French press. For example, malate dehydrogenase and fumarase were 30-40 per cent lower and the particulate enzymes (succinate dehydrogenase and cytochrome oxidase) were even more drastically affected. In contrast, the levels of the cytoplasmic enzymes hexokinase and catalase were little affected. These results suggested a selective loss of mitochondrial enzymes during preparation of cell fractions via the snail enzyme method. The stage at which this loss occurs has been investigated in detail (Vary, 1970) : it appears that mitochondria are centrifuged down when homogenates are spun to remove cell debris. This means that estimates of distributions are affected; the per cent mitochondrial content has to be considered as a minimum estimate.

TABLE A-2 a

PREINCUBATION CONDITIONS AND SNAIL ENZYME DIGESTION

Sample	1	2	3	4
mercaptoethanol, M	0.1	0.02	0.1	0.02
pH	9.3	8.0	8.0	9.3
snail digestion (%)	61	10	44	53

Cells were preincubated for 10 min at 30°. The snail digestion is expressed as the percentage decrease in $O.D._{640}$ after 45 min incubation. Distribution of enzymes after these treatments is shown in Table A-2 b.

TABLE A-2 b

DISTRIBUTION OF ENZYMES AND PROTEIN AFTER VARIOUS PREINCUBATION TREATMENTS

Sample	1		3		4	
	cyto.	mito.	cyto.	mito.	cyto.	mito.
cytochrome oxidase	23	77	16	84	7	93
succinate dehydrogenase	68	32	45	55	17	83
malate dehydrogenase	83	17	90	10	85	15
fumarase	84	16	83	17	80	20
catalase	99	1	98	2	98	2
protein	95.5	4.5	93	7	94	6

Results are expressed as the per cent of the total activity recovered in the two fractions. The sample numbers are the same as those in Table A-2 a, and define the preincubation conditions. Enzyme assays were carried out by Dr. M. Lowdon.

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List of Corrections / Additions

P 18 1 9	goldring
p 22 1 18	controversy
p 24 1 5	derepression
p 31 1 4 up	existence
p 35 1 8	Berkeley
p 37 1 16	vessel
p 42 1 11	coefficients
p 52 1 14	electron
p 54 1 6	sensitivities
p 62 1 9 up	derepressed
p 63 1 15 up	derepression
p 77 1 18	add 'of' between 'presence' and 'cycloheximide'
p 79 1 12	change 'supplemented' to'depleted'
p 98 1 2 up	lose
p 181 1 12 up	synthesis
p 191 1 18	phenazine
p 190 1 2 up	essentially
p 80 1 9 up	insert 'M' after '.05'
p 140 1 12	change 'allosteric' to 'allotropic'
p 194 1 3	change '0.3' to'9.3'

fig 4.3	add abscissa units and legend (see attached diagram)
fig 5.3	add abscissa units and legend (see attached diagram)
fig 4.10	add to legend "The experiment was carried out as detailed in Ch. II with ^{14}C-leucine (2 µCi/µmole) as precursor; 0.6 µCi was added to approx. 5 mg dry wt cells in 1.5 ml buffer"
fig 5.5	addition to legend, giving control values as requested; see attached sheet
fig 5.8	add to legend 'Results are those of Dr. M. Lowdon'.
fig 6.3	figure modified (see attached sheet)
fig 6.4	ordinate modified to read : 'cpm/mg dry wt'
Table 4.7	Heading of incorporation columns modified to read : 'leucine incorporation cpm/mg prot/15 min' Add to legend 'Incorporation conditions are described in detail in Chapter II'.

fig 4.3

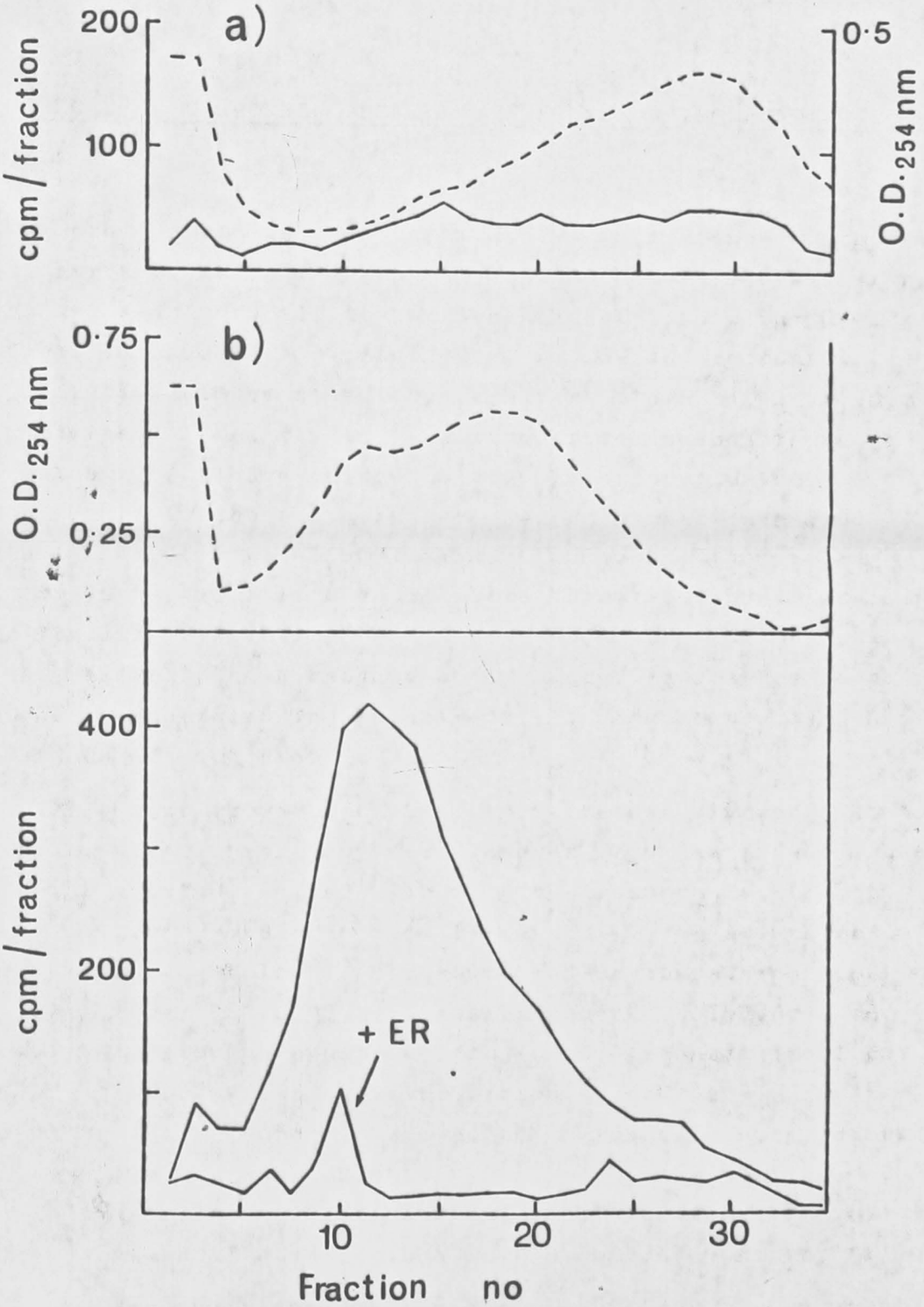
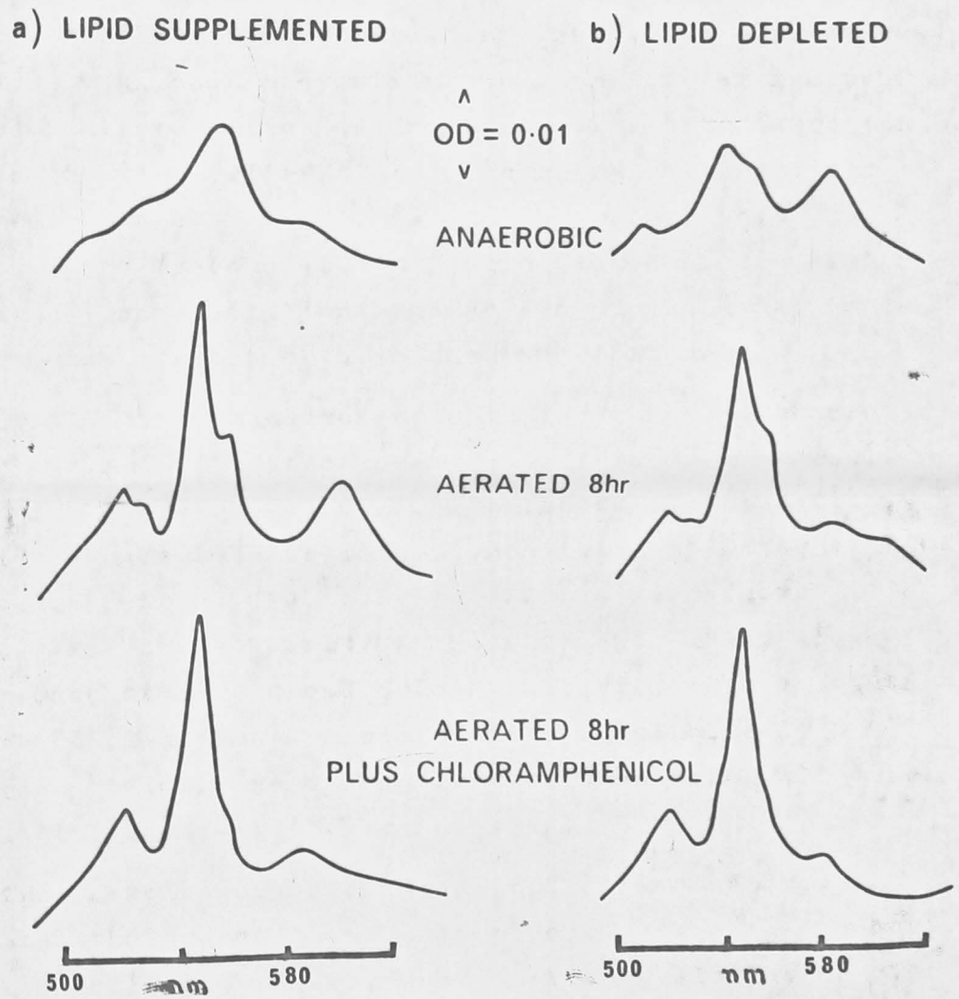

a)
200
100
cpm / fraction
0·5
O.D.254 nm
b)
0·75
0·25
O.D.254 nm
400
200
cpm / fraction
+ ER
10
20
30
Fraction no

Figure 5-3



Cytochrome content of cells grown anaerobically with or without lipid supplements, and after aeration in the presence or absence of chloramphenicol (10 mM).

figure 5-5

Effects of inhibitor concentration on the oxygen-induced synthesis of lipids and mitochondrial enzymes :

a) response to chloramphenicol;

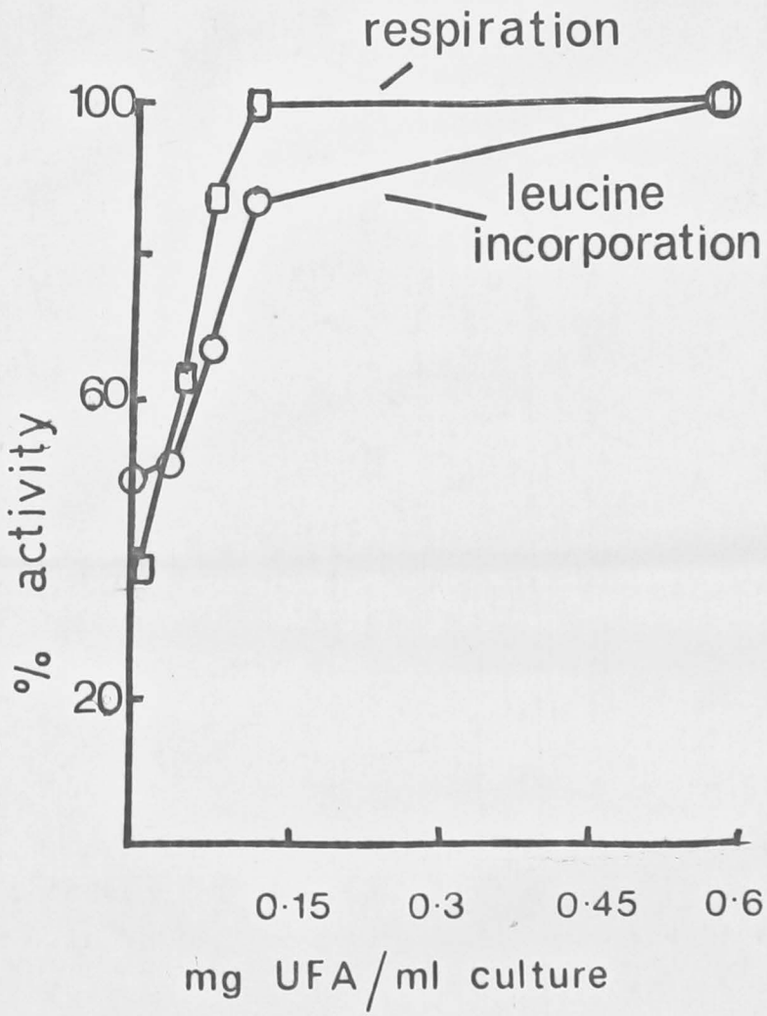
b) response to erythromycin.

Cells were grown anaerobically without lipid supplements for 20 hr, then aerated for 4 hr in the presence of the antibiotic concentration indicated. Cells were then analysed for lipid content, or broken in the Frence press for the preparation of a whole homogenate which was used for the enzyme assays; these assays were carried out by Dr. M. Lowdon.

▲	cytochrome oxidase	(0.108)
■	fumarase	(0.159)
□	succinate dehydrogenase	(0.140)
●	unsaturated fatty acid	(40)
O	ergosterol	(4.7)

Values in parentheses are control levels for the two experiments. The units of the enzyme activities are μmoles oxidized / min / mg protein, or O.D.U. change / min / mg protein in the case of fumarase : the lipid levels are expressed as mg / gm dry wt cells.

Figure 6-3



The development of respiration and protein-synthetic activity during aeration of anaerobically-grown, lipid-depleted KD115 cells; the response to unsaturated fatty acid (UFA) addition. The depleted cells were aerated in the presence of various amounts of UFA(as Tween 80). Glucose was pumped into the culture as described in the text. Results are expressed as per cent inhibitions relative to the activities found when 0.6 mg UFA/ml culture was used.